Bohn, Brent 1089

From: Hotchkiss, Andrew
Sent: Thursday, February 20, 2014 3:20 PM
To: Powers, Christina
Subject: RE:
Attachments: iAs and gonadotropins and ov steroidsf.pdf; aresenic and e2 signaling and uterus.pdf

From: Powers, Christina
Sent: Thursday, February 20, 2014 2:45 PM
To: Hotchkiss, Andrew
Subject: RE:

Thanks so much Andrew!

Christy

From: Hotchkiss, Andrew
Sent: Thursday, February 20, 2014 2:43 PM
To: Powers, Christina
Subject:

Here is the latest write-up. I have a call until 3 then will stop by to chat.

These data will have to be added to the tables...

A.

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EFFECT OF SODIUM ARSENITE ON PLASMA LEVELS OF GONADOTROPHINS AND OVARIAN STEROIDOGENESIS IN MATURE ALBINO RATS: DURATION-DEPENDENT RESPONSE

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ABSTRACT — Effect of arsenic on ovarian steroidogenesis at the dose available in drinking water at wide areas of West Bengal is reported here. Weights of ovary, uterus and vagina along with biochemical activities of ovarian Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) and plasma levels of LH, FSH and estrogen were measured in mature rats of the Wistar strain at diestrous phase following subchronic treatment with sodium arsenite at a dose of 0.4 ppm/rat/day for 16 days (4 estrous cycles) and 28 days (7 estrous cycles). A significant reduction in plasma levels of LH, FSH and estrogen along with significant diminution in the activities of ovarian Δ^5-3β-HSD and 17β-HSD were observed following sodium arsenite treatment for 28 days. This duration of treatment also resulted in a marked degree in diminution in the weights of ovary, uterus and vagina, but 16 days of treatment did not exhibit any significant effect on these above parameters. Arsenic-treated rats exhibited a prolonged diestrous phase in the estrous cycle in contrast to control rats having 4 days of a regular estrous cycle. Deposition of arsenic in ovary, uterus, vagina and plasma was also monitored in arsenic-treated rats. The results of our experiment suggest that duration of arsenic treatment is the critical factor for its adverse effect on ovarian activities at the dose within the range noted in drinking water at several areas of West Bengal in India.

KEY WORDS: Arsenic, Female sex organs, Ovarian steroid dehydrogenase, Estrogen, Gonadotrophic hormones

INTRODUCTION

Arsenic is a nonessential trace element. There are several reports which found that it is an important water pollutant, even present in drinking water in several countries, such as India [many districts of West Bengal] (Mazumder *et al.*, 1988; Saha, 1991), Bangladesh (Chatterjee *et al.*, 1995; Nickson *et al.*, 1998), Northern Chile, Thailand, Taiwan, China, Inner Mongolia, Mexico, Argentina, Finland and Hungary (Chappell *et al.*, 1997), and creating an epidemic of 'Arsenic Dermatosis' along with hyperkeratosis, gangrene and skin cancer (Mazumder *et al.*, 1988; Saha, 1995; Chowdhury *et al.*, 1997). This trace element is used frequently in herbicides, insecticides, rodenticides, food preservatives and as a byproduct of used fossil fuel (Baxley *et al.*, 1981; Flora *et al.*, 1995). Arsenic is also used for the treatment of syphilis, amoebiosis and certain other tropical diseases (Kawaguchi, 1981; Klaassen, 1990). Besides its clinical utility, arsenic intoxication is associated with severe metabolic disorders along with hepatic toxicity (Mahaffey *et al.*, 1981) and adrenal gland hypertrophy (Ghosh *et al.*, 1999), inhibition in testicular steroidogenesis (Sarkar *et al.*, 1991) as well as sper-

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matogenesis (Sukla and Pandey, 1984). Arsenic exposure also results in structural changes in the thymus of pregnant and newborn mice (Skal'naia *et al.*, 1995) and long-term exposure is associated with abortion, low birth weight and reduced lactation (Donald *et al.*, 1995). From the literature survey it has been revealed that there is lack of reporting related to the effect of arsenic on ovarian steroidogenic functions at the dose available in drinking water in wide areas of India and in other countries where this trace element is present at the range of 0.2 - 0.8 ppm in drinking water (Saha, 1991). Based on this literature survey, we have selected here the medium dose i.e. 0.4 ppm in connection with our previous experiment (Ghosh *et al.*, 1999), and this dose is present in drinking water at maximum zones of arsenic-polluted areas in India. Therefore, the results of this experiment will be very helpful to the community for general awareness of the toxic effects of arsenic on the female reproductive system.

MATERIALS AND METHODS

Animal selection and care

Forty adult female albino rats (8 weeks of age) of the Wistar strain having a regular 4-day estrous cycle and weighing 150 g-160 g were selected for this experiment. Animals were maintained under standard laboratory conditions (14hr light: 10hr darkness and 30±2℃) with free access to food and water. The Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) were followed throughout the experimental schedule.

Arsenic treatment, study of estrous cycle and sample collection

To explore the minimal effective duration of sodium arsenite on ovarian function, animals were divided into four groups. Their initial body weights were recorded and volume of daily water intake of each animal was studied for 10 days prior to experimentation, and this was 10 ml. Each control animal was provided with 10 ml distilled water/day and each animal of the treated group was provided with 10 ml distilled water containing Na-arsenite at the concentration of 0.4 ppm for 16 days and 28 days according to the experimental schedule. Vaginal smear was collected twice daily (8 A.M. and 4 P.M.) from all animals. Smears were stained by hematoxylin-cosine and observed under the microscope. Feeding habits of all the animals were observed throughout the experimental schedule. All the animals were sacrificed by decapitation at the diestrous phase after 20 hr of the last arsenic treatment. Body weights of all the animals were also recorded on the day of sacrifice. Blood was collected from the dorsal aorta using a heparinized syringe (21- gauge needle). Plasma samples were separated by centrifugation and stored at –20℃ until all the samples had been used for the determination of plasma levels of gonadotrophins, estrogen and arsenic. Ovary, uterus and vagina were dissected out and organs' weights were measured by single pan electronic balance. All these organs were used to monitor the level of arsenic deposition by atomic absorption spectrometry (Varian AA 575 ABQ, USA). Ovaries from each animal were also used to assay the activities of steroidogenic enzymes in a biochemical manner.

Assay of ovarian Δ^5-3β- and 17β-hydroxysteroid dehydrogenase activities

To study ovarian Δ^5-3β- hydroxysteroid dehydrogenase (Δ^5-3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities, one ovary from each animal was homogenized, maintaining a chilling condition at 4℃ in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA at a tissue concentration of 10 mg/ml homogenizing mixture with a homogenizer (Remi R Q - 127A, Mumbai, India). This mixture was centrifuged at 10,000 g for 30 min at 4℃ in a cold centrifuge (Beckman, Avanti TM30, USA). The supernatant (1 ml) was mixed with 100 μM sodium phosphate buffer (pH 8.9) and 30 μg of dehydroepiandrosterone (DHEA) making an incubation mixture of a total of 3 ml. Δ^5-3β-HSD activity was measured after addition of 0.5 μM of NAD to the tissue supernatant mixture in a spectrophotometer (U-2001, Hitachi, Tokyo, Japan) at 340 nm against a blank (without NADP) (Talalay, 1962). For ovarian 17β-HSD activity measurement, the supernatant fluid (1 ml) of the homogenizing mixture was collected the same way and 440 μM of sodium phosphate buffer (pH 10.2), 25 mg crystalline BSA and 0.3 μM testosterone was added, making the incubation mixture a total 3 ml. Enzyme activity was measured after addition of 1.1 μM NADP to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NADP) (Jarabak *et al.*, 1962). One unit of enzyme activity is equivalent to a change in absorbancy of 0.001 / min at 340 nm.

Radioimmunoassay (RIA) of LH, FSH

Plasma levels of LH and FSH were measured by RIA (Moudgal and Madhwa Raj, 1974) using reagents supplied by the Rat Pituitary Distribution Program and NIDDK (Bethesda, MD). Carrier free ^{125}I for hormone

iodination was obtained from Bhaba Atomic Research Center (Mumbai, India). Pure rat FSH (NIDDK-rFSH-I-5) and LH (NIDDK-rLH-I-5) were iodinated using the Chloramine-T (Sigma Chemical Co., St.Louis, MO) (Greenwood *et al.*, 1963). NIDDK anti-rat-FSH-S-11 and NIDDK anti-rat-LH-S-5 were used as anti-sera at final dilutions of 1: 2,500 and 1: 10,000, respectively. Goat anti-rabbit γ-globulin was used as the second antibody. It was obtained from Indo-Medicine (Friendswood, TX). The intra-assay variations were 6% and 5% for FSH and LH, respectively. All samples were run in one assay to avoid inter-assay variation.

Radioimmunoassay (RIA) of estrogen

Plasma level of estrogen was assayed by RIA (Hanning *et al.*, 1974). Methodological loss during extraction was monitored by adding 10,000 cpm (1β, 2β-3H(N)) estradiol before extraction with 4 ml of diethyl ether twice. Samples were assayed in duplicate. The anti-sera to estradiol was purchased from Endocrine Science (Tarzana, CA), and it has a 40% cross-reactivity with estrone. Free and bound estradiol were separated by using dextran-coated charcoal. The intra-assay variation was 6.5%. All samples were run at one time to avoid inter-assay variation. Since a chromatographic purification of the samples was not performed, the values reported are the sum of estradiol and estrone.

Determination of elementary arsenic content

Arsenic contents in plasma and ovarian, uterine, vaginal tissues were measured by atomic absorption spectrometry (Locke, 1979; Nurenberg, 1982; Imelda *et al.*, 1996). Thirty mg of ovarian tissue, 50 mg of uterine and vaginal tissues, and 0.5 ml of plasma were fixed in formaldehyde and dried at 150℃ for 20 min to evaporate the formaldehyde. Next, samples were transferred inside quartz beakers which had been washed with 1 : 1 HNO_3 and H_2SO_4 solution. Samples were digested with 2 ml pure nitric acid mixture inside a digestion chamber at 150℃ for 20 min. Until a small volume was obtained, beakers were capped and enclosed samples were boiled. Digested samples were filtered and diluted up to 25 ml with de-ionized distilled water. The same de-ionized water was used as blanks and a reading taken in the Varian AA 575 ABQ (USA) model of the atomic absorption spectrometer.

Statistical analysis

Statistical significance of the differences in these variables between treated cases and controls were evaluated using the two-tailed Students *t* test (Zar, 1996). Differences of data (Mean±SE, N=10), $p< 0.05$ were considered significant statistically.

RESULTS

Feeding habit, body growth and organ weights

Animals of all groups received a diet at a normal level throughout the experimental schedule, and there was no change in the volume of water intake per animal in each group throughout the duration of the experiment. Body weight of arsenic-treated animals did not differ significantly from that of the control (Table 1). No significant alteration was noted in the wet weights of ovary, uterus and vagina after 16 days of arsenic treatment in respect to control. After 28 days of arsenic treatment, there was a significant inhibition in the wet weight of these sex organs in comparison to the respective control group (Table 1).

Vaginal smear study

In control groups regular four-day estrous cycles were noted, whereas in the case of the treated group a constant diestrous stage was observed just after 18±2 days of arsenic ingestion.

Ovarian Δ^5-3β-HSD and 17β-HSD activities

Twenty-eight days of arsenic treatment exhibited a significant inhibition in ovarian Δ^5-3β-HSD and 17β-HSD activities in comparison with controls, but no significant change was observed in these enzymatic activities after 16 days of arsenic exposure in respect to the control group (Fig. 1).

Plasma FSH, LH and estrogen

Arsenic treatment for 28 days resulted in a significant reduction in plasma levels of LH, FSH and estrogen (Fig. 2) in comparison to controls, whereas 16 days of treatment did not alter the plasma levels of these hormones significantly.

Arsenic content in plasma and sex organs

Elementary arsenic decomposition in plasma, ovary, uterus and vagina was monitored and the levels of arsenic in all these tissues of treated animals for both durations were significantly elevated when compared with the control group (Table 2).

DISCUSSION

These results provide evidence for the adverse effect of sodium arsenite on the pituitary-ovarian axis when the

level of arsenic in water is within the range of arsenic-polluted drinking water noted in different areas of West Bengal in India. The term of 28 days (7 estrous cycles) of arsenic treatment is the critical duration that affects the pituitary-ovarian axis. Inhibition in the activities of ovarian Δ^5-3β-HSD and 17β-HSD in rats in this experiment may be due to the result of low levels of plasma LH and FSH, since these gonadotrophins are responsible for regulating the activities of these steroidogenic enzymes (Odell *et al.*, 1963). Plasma levels of estrogen were decreased, and the decreases may be due to the inhibition of Δ^5-3β-HSD and 17β-HSD activities in the ovary, as these are rate-limiting key enzymes for ovarian steroidogenesis (Hinshelwood *et al.*, 1994). Diminution in plasma levels of LH and FSH in arsenic-treated rats may be due to the hypersecreation of ACTH and glucocorticoids (Ghosh *et al.*, 1999), since the high level of ACTH in plasma inhibits gonadotrophin secretion (Christian, 1964; Ogle, 1977) and elevation in plasma levels of glucocorticoids inhibits the sensitivity of gonadotrophin cells to the gonadotrophin-releasing hormone (GnRH), as well as suppressing gonadotrophin secretion (Luton *et al.*, 1977; Ringstrom and Schwartz, 1985; Kamel and Kubajak, 1987).

The diminution in ovarian, uterine and vaginal weights in arsenic-treated rats may be due to low plas-

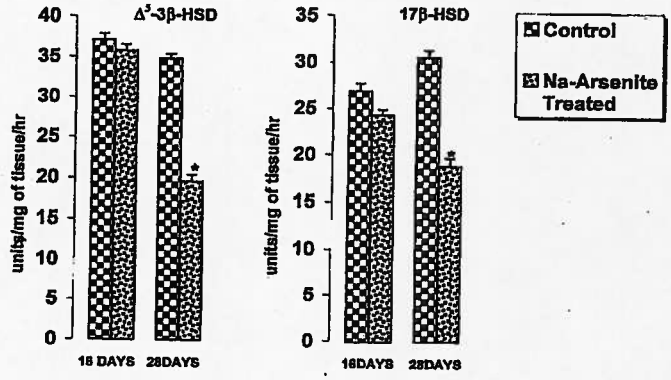

Fig. 1. Changes in ovarian Δ^5-3β and 17β- hydroxysteroid dehydrogenase activities in rats treated with 10 ml water/day containing Na-arsenite at the concentration of 0.4 ppm for 16 and 28 days. Controls were provided with 10 ml water/day for the same duration. Data are expressed as mean±SE (N=10). An asterisk indicates an experimental value significantly different from the respective control value ($p< 0.001$).

Table 1. Changes in body weight, and ovarian, uterine and vaginal weights after arsenic treatment in rats.

GROUP	Initial body wt. (g)	Final body wt. (g)	Ovarian wt. in pair (mg)	Uterine wt. (mg)	Vaginal wt. (mg)
Control (16 days)	151±6	168±7	96.3±5.2	134.7±7.3	143.8±7.1
Na-arsenite treated (16 days)	154±8	165±9	83.5±5.4	119.6±7.4	126.7±6.4
Control (28 days)	157±7	172±5	94.8±4.5	155.3±7.1	153.6±7.2
Na-arsenite treated (28 days)	159±7	169±6	55.2±5.1*	102.2±7.4*	81.5±7.5*

Rats were treated with 10 ml water/day containing Na-arsenite at the concentration of 0.4 ppm for 16 and 28 days. Controls were provided with 10 ml water. Mean±SE, N=10, * $p < 0.001$.

ma levels of LH, FSH and estrogen, since ovarian weight is regulated by gonadotrophins (Tagatz *et al.*, 1970; Kulin and Reiter, 1973), whereas uterine and vaginal weights are under the regulation of estrogen (Edman, 1983).

The duration-dependent adverse effect of arsenic on ovarian activities reflects the mode of action of arsenic on the pituitary-ovarian axis. A 28-day arsenic exposure exerted detrimental effects on ovarian steroidogenesis and resulted in marked diminution in plasma levels of LH, FSH and estrogen along with low ovarian, uterine and vaginal weights, though 16 days of treatment had no such effect on these above parameters. Therefore, it may be suggested that arsenic possibly has no direct effect on ovarian activities. Consistent diestrous in arsenic-treated rats after 18±2 days may be due to low plasma levels of estrogen (Parshad *et al.*, 1989) and it also indicates the indirect effect of arsenic on the ovary i.e. via the pituitary-ovarian axis. As there was no significant alteration in body weight of the treated animals with respect to controls, these adverse effects of arsenic on the ovary were not due to its general toxic effect but may be due to its toxicity on target organs.

In conclusion, the results presented here provide evidence that arsenic treatment is associated with a

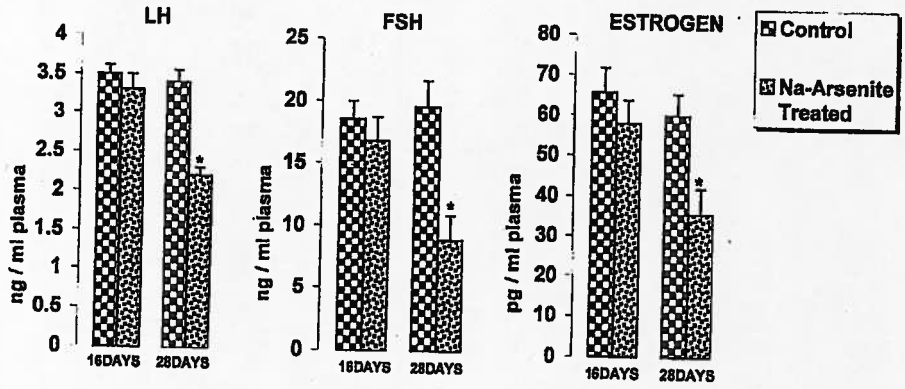

Fig. 2. Changes in plasma levels of LH, FSH and estrogen in rats treated with 10 ml water/day containing Na-arsenite at the concentration of 0.4 ppm for 16 and 28 days. Controls were provided with 10 ml water/day for the same duration. Data are expressed as mean ± SE (N=10). An asterisk indicates an experimental value significantly different from the respective control value ($p < 0.001$).

Table 2. Elementary arsenic content in ovary, uterus, vagina and plasma before and after arsenic treatment.

GROUP	ARSENIC CONTENT			
	Ovary (μg/g)	Uterus (μg/g)	Vagina (μg/g)	Plasma (μg / 100 ml)
Control (16 days)	2.52±0.21	3.50±0.18	0.45±0.08	0.336±0.005
Na-arsenite treated (16 days)	5.80±0.2*	9.50±0.35*	1.32±0.14*	2.12 ±0.01*
Control (28 days)	2.57±0.3	3.55±0.22	0.50±0.1	0.40 ±0.009
Na-arsenite treated (28 days)	6.60±0.15*	11.43±0.25*	1.46±0.1*	2.41 ±0.01*

Rats were treated with 10 ml water/day containing Na-arsenite at the concentration of 0.4 ppm for 16 and 28 days. Controls were provided with 10 ml water. Mean ± SE, N=10, * $p < 0.001$.

reduction in the activities of ovarian steroidogenic dehydrogenase, low plasma levels of LH, FSH and estrogen, and diminution in the weights of uterus, ovary and vagina. In addition, our data suggest an effect of arsenic at the pituitary level. If arsenic would have acted only at the ovarian axis, then we would have increased levels of plasma gonadotrophins after 16 days of treatment by the withdrawal of a negative feedback effect on the pituitary by estrogen. Moreover, our experimental data also support the point that ovarian activity is affected by sub-chronic arsenic treatment when the level of arsenic in drinking water is within the range found at wide areas of West Bengal, India. Therefore, these results provide important conceptual information concerning possible adverse effects of arsenic on the ovarian function, and our data have an applied value in environmental toxicology. However, more information is necessary to better understand the effect of arsenic on the functional physiology of the female reproductive system.

REFERENCES

Baxley, M.N.; Hood, R.D., Vedel, G.C., Harison, W.T. and Szczech, G.M. (1981): Prenatal toxicity of orally administered sodium arsenite in mice. Bull. Environ. Contam. Toxicol., **26**, 749-756.

Chappell, W.R., Beck, B.D., Brown, K.G., Chaney, R., Richard, C.C., Irgolic, K.J., North, D.W., Thornton, I. and Tsongas, T.A. (1997): Inorganic arsenic: A need and an opportunity to improve risk assessment. Env. Health. Perspec., **105**, 1060-1065.

Chatterjee, A., Das, D., Mandal, B.K., Samanta, G. and Banerjee, P.(1995): The biggest arsenic calamity in the world. Analyst, **120**, 643-650.

Chowdhury, T.R., Mandal, B.K., Samanta, G., Basv, G. K., Chowdhury, P.P., Chanda, C.R., Karan, N.K., Lodh, D., Dhar, R.K. and Das, D. (1997): Arsenic in ground water in six districts of West Bengal, India-The biggest arsenic calamity in the world: The status report up to August, 1995. In Arsenic Exposure and Health effects. (Abernathy, C.O., Calderson, R.L. and Chappell, W. R. eds.), pp. 93-111, Chapman and Hall, New York.

Christian, J.J. (1964): Actions of ACTH in intact and corticoid-maintained adrenalectomized female mice with emphasis on the reproductive tract. Endocrinology, **75**, 655-657.

Donald, M.C., Edwards, R.A. and Greenhalgh, J.F.D. (1995): Minerals. In Animal Nutrition. (Donald, M.C., Edwards, R.A. and Greenhalgh, J.F.D. eds.), pp. 127-136, ELBS, England (Longman).

Edman, C.D. (1983): The effect of steroid on endometrium. Semin. Reprod. Endocrinology, **1**, 179-187.

Flora, S.J.S., Dube, S.N., Arora, U., Kannan, G.M., Shukla, M.K. and Malhotra P.R. (1995): Therapeutic potential of meso 2,3-dimercaptosuccinic acid or 2,3-dimercaptopropane 1-sulfonate in chronic arsenic intoxication in rats. Bio. Metals, **8**, 111-116.

Ghosh, D., Chattopadhyay, S. and Debnath, J. (1999): Effect of sodium arsenite on adrenocortical activity in immature female rats: Evidence of dose-dependent response. J. Environ. Sci. **11**, (in press).

Greenwood, F.O., Hunter, W.M. and Glover, J.S. (1963): The preparation of ^{131}I labeled human growth hormone of high specific activity. Biochem. J., **89**, 114-123.

Hanning, R., Oreyk, P.J., Caldwell, V.B. and Behrman, H.R. (1974): Estrogen and its radio-immunoassay. In Methods of Hormone Radio- immunoassay. (Jaffe, B. M. and Behrman, H. R. eds.), pp. 675-685, Academic Press, New York.

Hinshelwood, M.M., Demter-Arlotto, M., Means, G. D. and Simpson, E.R. (1994): Expression of genes encoding steroidogenic enzymes in the ovary. In Molecular Biology of the Female Reproductive System. (Findlay, J.K. ed.), pp.129-145, Academic Press, London.

Imelda, F.T., Saldivar, O.L., Tovar, T.A., Salazar, D. and Olaiz, F.Z. (1996): Metals in lung tissue from autopsy cases in Mexico City residents: Comparison of cases from the 1950s and the 1980s. Env. Health Perspec., **6**, 630-632.

Jarabak, J., Adams, J.A., Williams-Ashman, H.G. and Talalay, P. (1962): Purification of 17β- hydroxysteroid dehydrogenase function. J. Biol. Chem., **237**, 345-357.

Kamel, F.A. and Kubajak, C.L. (1987): Modulation of gonadotropin secretion by corticosterone interaction with gonadal steroids and machanism of action. Endocrinology, **121**, 561-565.

Kawaguchi, I. (1981): Studies on As_2O_3-induced hyperglycemia. NipponYakurigaku Zasshi- Folia Pharmacol. Japon., **78**, 213-222.

Klaassen, C.D. (1990): Heavy metals and heavy metal antagonists. In The Pharmaceutical Basis of Therapeutics. (Goodman, G.A., Rall, T.W., Nies, A.S. and Taylor, P. eds.), pp. 1602-1605, Pergamon Press, New York.

Kulin, H.E. and Reiter, E.O. (1973): Gonadotrophins dur-

ing childhood and adolescence. A review, Pediatrics, **51**, 260-271.

Locke, J. (1979): The determination of eight elements in human liver tissue by flame atomic absorption spectrometry in sulfuric acid solution. Anal. Chem. Acta., **104**, 225-231.

Luton, J., Thiebott, P., Valcke, J., Mahendean, J.A. and Bricaire, H. (1977): Reversible gonadotropin deficiency in male Cushing disease. J.Clin. Endocrinol. Metab., **45**, 488- 493.

Mahaffey, K.R., Capar, S.G., Gladen, B.C. and Fowler, B.A. (1981): Concurrent exposure to lead, cadmium and arsenic: Effects of toxicity and tissue metal concentration in the rat. J. Lab. Clin. Med., **98**, 463-481.

Mazumder, D.N., Guha, A., Chakraborty, A.K. and Ghosh, A. (1988): Chronic arsenic toxicity from drinking tube-well water in rural West Bengal. Bull. WHO., **66**, 499-506.

Moudgal, N.R. and Madhwa Raj, H.G. (1974): Pituitary gonadtrophin. In Methods of Hormone Radioimmunoassay. (Jaffe, B. M. and Behrman, H. R. eds.), pp. 57-85, Academic Press, New York.

Nickson, R., McArthur, J., Burges, W., Ahmed, K.M., Ravenserof, P. and Rahman, M. (1998): Arsenic poisoning of Bangladesh ground water. Nature, **395**, 338.

Nurenberg, W.H. (1982): Voltametric trace analysis in ecological chemistry of toxic metals. Pure Appl. Chem., **54**, 853-878.

Odell, W.D., Swerdloff, R.S., Bain, J., Wallesen, F. and Grover, P. K. (1963): The effect of sexual maturation testicular response to LH stimulation of testosterone secretion in the intact rat. Endocrinology, **72**, 452-464.

Ogle, T.F. (1977): Modification of serum leuteinizing hormone and prolactin concentration by corticotropin and adrenalectomy in ovariectomized rats. Endocrinology, **101**, 494-497.

Parshad, V.R., Kaur, P. and Guraya, S.S. (1989): Reproductive cycles of mammals: Rodentia. In Reproductive Cycles of Indian Vertebrates. (Saidapur, S. K. ed.), pp.347- 408, Allied Publishers Ltd., Bombay.

Ringstrom, S.J. and Schwartz, N.B. (1985): Cortisol suppresses the LH release and ovulation in cyclic rat. Endocrinology, **116**, 472-475.

Saha, A.K. (1991): Pollution in ground water in West Bengal: Final report, Steering Committee, Arsenic Investigation Project, PHE Dept., pp. 1-56, Government of West Bengal.

Saha, K.C. (1995): Chronic arsenical dermatosis from tube-well water in West Bengal during 1983-1987. Indian J. Dermatol., **40**, 1-12.

Sarkar, M., Biswas, N.M. and Ghosh, D. (1991): Effect of sodium arsenite on testicular Δ^5-3β, 17β-HSD activities in albino rats: Dose- and duration-dependent responses. Med. Sci. Res., **19**, 789-790.

Skal'naia, M.G., Zhavoronkov, A.A. and Skal'nyi, A.V. (1995) : Morphologic characteristics of the thymus in pregnant and newborn mice. Arkhiv. Patologii, **57**, 52-58.

Sukla, J.P. and Pandey, K. (1984): Impaired spermatogenesis in arsenic-treated fresh water fish *Colisa fasciatus* (Bl & Sch). Toxicol. Lett., **21**, 191-195.

Tagatz, G.E., Fialkow, P.J. and Smith, D. (1970): Hypogonadotropic hypogonadism associated with anosmia in the female. N. Engl. J. Med., **283**, 1326-1329.

Talalay, P. (1962): Hydroxysteroid dehydrogenase. In Methods in Enzymology. (Colowick, S. P. and Kaplan, N. O. eds.), vol. 5 , pp. 512-516, Academic Press, New York.

Zar, J.H. (1996): One sample hypothesis. In Biostatistical Analysis. (Zar, J. H. ed.), pp. 93-98, Prentice Hall, New Jersey.

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REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

RESEARCH Open Access

Arsenic abrogates the estrogen-signaling pathway in the rat uterus

Aniruddha Chatterjee and Urmi Chatterji*

Abstract

Background: Arsenic, a major pollutant of water as well as soil, is a known endocrine disruptor, and shows adverse effects on the female reproductive physiology. However, the exact molecular events leading to reproductive dysfunctions as a result of arsenic exposure are yet to be ascertained. This report evaluates the effect and mode of action of chronic oral arsenic exposure on the uterine physiology of mature female albino rats.

Methods: The effect of chronic oral exposure to arsenic at the dose of 4 microg/ml for 28 days was evaluated on adult female albino rats. Hematoxylin-eosin double staining method evaluated the changes in the histological architecture of the uterus. Circulating levels of gonadotropins and estradiol were assayed by enzyme-linked immunosorbent assay. Expression of the estrogen receptor and estrogen-induced genes was studied at the mRNA level by RT-PCR and at the protein level by immunohistochemistry and western blot analysis.

Results: Sodium arsenite treatment decreased circulating levels of estradiol in a dose and time-dependent manner, along with decrease in the levels of both LH and FSH. Histological evaluation revealed degeneration of luminal epithelial cells and endometrial glands in response to arsenic treatment, along with reduction in thickness of the longitudinal muscle layer. Concomitantly, downregulation of estrogen receptor (ER alpha), the estrogen-responsive gene - vascular endothelial growth factor (VEGF), and G1 cell cycle proteins, cyclin D1 and CDK4, was also observed.

Conclusion: Together, the results indicate that arsenic disrupted the circulating levels of gonadotropins and estradiol, led to degeneration of luminal epithelial, stromal and myometrial cells of the rat uterus and downregulated the downstream components of the estrogen signaling pathway. Since development and functional maintenance of the uterus is under the influence of estradiol, arsenic-induced structural degeneration may be attributed to the reduction in circulating estradiol levels. Downregulation of the estrogen receptor and estrogen-responsive genes in response to arsenic indicates a mechanism of suppression of female reproductive functions by an environmental toxicant that is contra-mechanistic to that of estrogen.

Background

Arsenic is a naturally occurring metalloid with potent toxic and mutagenic effects [1]. It is present ubiquitously in the environment and is released from both natural and man-made sources [2]. Arsenic in drinking water is one of the topmost environmental threats worldwide, based on the potential exposure of people to arsenic and the numerous diseases with which it has been associated [3-6]. In Southeast Asian countries like India, Bangladesh and Taiwan, millions of people are threatened by arsenic poisoning, leading to several diseases and disorders, and even death [7]. The problem of arsenic poisoning is not only restricted to developing countries but developed nations like USA, Germany, China, Japan and Australia are also plagued by problems of arsenic contamination [8,9]. Interestingly, inorganic arsenic is found to be more potent than the organic form and trivalent compounds are found to be more toxic than pentavalent ones [10].

Chronic intake of arsenic is strongly associated with an increased risk of skin, lung, liver and other cancers, type 2 diabetes, cardiovascular diseases, neurological and cognitive defects, and reproductive and developmental problems [11-16]. According to World Health Organization, the permissible limit of arsenic in drinking water is 0.01 mg/l, which is equivalent to 10 ppb [9,15,17,18]. Recently, however, it has been reported that there is an increased

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risk of arsenic toxicity, even at the low and permissible dose of 10 ppb [9,15-17]. However, a large population all over the world is exposed to far higher levels of arsenic [19-23]. In certain areas in the Indian subcontinent, the maximum arsenic concentration in ground water was found to be around 3700 ppb [24] to 4700 ppb [18], leading to several physiological damages to human beings. Although arsenic is not a direct acting xenotoxin or mutagen, it may increase DNA damage or mutations indirectly by altering DNA repair, thus acting as a co-carcinogen or promoter of tumor growth [25].

Till date, there is very little information regarding the mechanism of arsenic action on the ovarian steroidogenic function and the female reproductive axis, particularly in wide areas of India and other countries, where the levels occurring in drinking water exceed the admissible limits of 10 ppb [20-22,24]. It is, however, known that women who are exposed to this level of arsenic often suffer from spontaneous abortion and stillbirth [26], and maternal exposure to arsenic also affects the health of newborn and promotes carcinoma incidences in them [27-29]. Although it has been hypothesized that the reproductive hazards may be due to disruption of the steroid hormone signaling pathway [30,31], the actual target of arsenic is probably a part of the mechanism which is used to regulate gene expression and not just the receptor itself [31]. The estrogen receptor (ER) is the most divergent of all steroid hormone receptors and may undergo divergent co-regulatory interactions and unique activation/de-activation steps. Previous studies have suggested that arsenic can interfere with ER functioning, although the exact mechanism remains to be ascertained [31]. Since arsenic acts as a potent environmental estrogen [32], it was explicable to study the arsenic-stimulated estrogen receptor signaling pathway, expression of estrogen responsive genes such as VEGF, and G1 cell cycle regulatory proteins CDK4 and cyclin D1, since these molecules are known to be primary responsive factors to estrogen administration in the rat uterine endometrium [33-35]. Thus, the objective of this study was to elucidate the role of arsenic as an endocrine disruptor and determine the molecular mechanism underlying arsenic action in the rat uterus.

Methods

Animals

Female Sprague-Dawley rats, aged 15-16 weeks and weighing 100-120 g, were collected from the breeding colony and maintained under controlled conditions (25 ± 2°C temp, 50 ± 15% RH and normal photoperiod 12 h dark and 12 h light) through out the experiment. The animals were given sterile food pellets and water *ad libitum* and allowed to acclimatize to the laboratory environment for 5 days prior to the commencement of the experiments. The Principles of Laboratory Animal Care (NIH Publication No 85-23, revised in 1985) as well as specific Indian Laws of Animal Protection (ILAP) were followed through out the experimental schedule.

Drug treatments, selection of optimum dose and time and study of estrous cycle

Sodium arsenite (E-Merck, Germany) was used for study. All other chemicals were procured from Sigma Aldrich (USA). The rats were divided randomly into different groups, each containing 5 animals. Group I (control group) animals were fed 10-12 ml pure distilled water/animal/day, while other groups were fed the same volume of water containing sodium arsenite at different concentrations (0.4 μg/ml, 4 μg/ml, 40 μg/ml and 80 μg/ml) [27-29,36-38] and maintained for different time periods (7 days, 14 days, 28 days and 56 days) in order to determine the optimum dose and time of arsenic action. The optimum time and dose of arsenic action, as determined by circulating estradiol concentrations, were selected for all subsequent experiments. The dose of arsenic thus selected conforms to environmentally relevant concentrations. Vaginal smears were collected every morning from all of the animals before fresh treatment of arsenic. The smears were double stained with eosin-hematoxylin and examined microscopically.

Tissue and blood collection

Animals were anesthetized by intraperitoneal injection of sodium barbital. Uteri were quickly removed from the experimental animals and washed in 0.9% (w/v) cold normal saline, pat dried and weighed in an electrical monopan balance (Lutron GM-300 P). Small representative tissue slices were processed for histological and immunohistochemical studies, RNA isolation and protein purification. Blood was collected from the heart and serum was isolated for ELISA.

Assay of serum estradiol, LH and FSH

ELISA (DRG International ELISA Kit) was performed for estimating the circulating levels of estradiol, LH and FSH. For assay of serum estradiol, 25 μl each of standard, control and treated serum samples were added to respective wells coated with anti-estradiol antibody and incubated with 200 μl of enzyme conjugate for 2 hours at room temperature. Subsequently, 100 μl of substrate was added and incubated for 15 minutes at room temperature. Reactions were stopped using 50 μl of stop solution and the O.D. was measured at 450 nm. Each sample was run in triplicate [39]. For assay of serum FSH and LH, 25 μl of standard, control or treated serum samples were added to respective wells coated with anti-FSH and anti-LH antibodies and incubated with 100 μl of enzyme conjugate for 30 minutes at room temperature. Wells were washed with *aqua dest* and 100 μl of substrate was added to each well. After incubation for 10 minutes at room temperature,

reactions were stopped using 50 µl of stop solution and the O.D. measured at 450 nm [38,39]. The intra-assay and inter-assay variations were found to be less than 9% and 10%, respectively. Limit of detection for estradiol was 3.6 pg/ml, for LH was 0.45 mIU/ml and for FSH was 0.28 mIU/ml.

Histology and morphometric analysis

Uterine slices (selected randomly from the proximal, middle and distal regions of the uterus) from control and treated animals were fixed in bouins fluid. Graded dehydration of the tissue was done by 70 to 100% alcohol in subsequent steps and xylene was used as the clearing agent. For histological studies, 5 µ paraffin sections were stained by standard hematoxylin-eosin double staining procedure and observed under a microscope [31]. The stained sections were subjected to morphometric analysis using the eye piece scale (occulometer) and the stage micrometer. The stage and the eye piece scales were adjusted until there was a parallel point between the two scales. The number of the eye piece divisions and its corresponding stage measurements was noted. The occulometer fixed into the microscope was then focused through stained sections of the tissue to allow for measurement of the luminal diameter, height of luminal epithelial cells, size of endometrial glands and longitudinal muscle layer [40].

Immunohistochemistry

Immunohistochemistry was performed according to a previously established protocol [26]. Briefly, sections were deparaffinized, hydrated, boiled in 0.1 M sodium citrate buffer (pH 6.0) for 10 minutes and treated with 3% (v/v) H_2O_2 in PBS (30 min) to block endogenous peroxidase activity. Non-specific staining was blocked by incubating the sections with bovine serum at room temperature. Sections were subsequently incubated with rabbit anti-ER IgG (sc-542, Santa Cruz Biotech, CA, 1:500) for 18 hours at 4°C and then with goat anti-rabbit HRP-conjugated secondary antibody (1:1000) for 1 hour at room temperature. Peroxidase was visualized using 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin. Slides that served as negative controls were not incubated with the primary antibody [27].

RNA extraction and semi-quantitative RT-PCR

Uterine tissues were collected from the arsenic-treated and untreated rats. 100 mg of tissue samples were frozen quickly in liquid nitrogen and total RNA was isolated using TRI reagent (SIGMA), dissolved in DEPC water and quantified by UV spectrophotometry. The RNA samples were subjected to DNase treatment prior to RT-PCR. Reverse transcription reaction was performed at 42°C with 5 µg of RNA in 5× reaction buffer (Fermentas, USA) containing 100 pmol random hexamer primer, 10 mM dNTP mixture (Fermentas, USA), 20 units of RNase inhibitor (Bioline, USA) and 200 units of RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, USA). PCR was initiated using 2.5 µg cDNA, 10 mM dNTPs and 1 unit of Taq DNA Polymerase (Vivantis, USA). PCR amplifications were performed using the primers listed in Table 1[41-45]. PCR was carried out for 40 cycles using an annealing temperature of 58°C for ERα and 55°C for VEGF, CDK4 and cyclin D1. Samples were fractioned by 2% agarose gel electrophoresis and quantified using a BioRad Gel Documentation System.

Western blot analysis

Tissue samples were lysed in ice-cold RIPA Buffer (150 mM NaCl, 50 mm Tris, 0.1% Triton X-100 and 0.1% SDS containing protease inhibitors [4-(-2-aminoethyl benzenesulphonyl fluoride), EDTA, leupeptin, aprotinin and bestatin, SIGMA]. The concentration of total protein was determined by Bradford assay and equal amount of proteins (30 µg) were fractioned by 8% SDS-PAGE. Proteins were electrically transferred to PVDF membranes and blocked for 2 hours at room temperature with 5% non-fat dry milk. Blots were subsequently incubated with ERα, VEGF and CDK4 antibodies raised in rabbit and cyclin D1 antibody raised in mouse (1:1000), for 18 hours at 4°C. Anti-β tubulin was used as a loading control. Blots were subsequently incubated with HRP-conjugated goat anti-rabbit and goat anti-mouse IgG secondary antibodies (1:2000), respectively, for 1 hour at 25°C. Immunoreactive proteins were detected by staining the membranes with 3,3'-diaminobenzidine in 50 mM Tris (pH-7) containing 0.2% H_2O_2 [46] and bands were quantified by a BioRad Gel Documentation System.

Table 1: Sequences of the oligonucleotides used for semi-quantitative RT-PCR

Gene	Forward primer	Reverse primer	Ref.
ER α	GGAGACATGAGAGCTGCCAAC	CCAGCAGCATGTCGAAGATC	[41]
VEGF	GATCAAGTTCATGGACGTCT	GATCAAGTTCATGGACGTCT	[43]
Cyclin D1	CTGGCCATGAACTACCTGGA	GTCACACTTGATCACTCTGG	[45]
GAPDH	GACATCAAGGTGGTGAAGCAG	CACCCTGTTGCTGTAGCCATATTC	[42]
CDK4	TGGTGTCGGTGCCTATGGGA	GGTAGCTGTAGATTCTGGCT	[44]

Statistical analysis

Results of the experiments performed in triplicates were expressed as mean and standard error of mean of different groups, using a statistical software package (Graphpad). The differences between the mean values were evaluated by one-way ANOVA followed by multiple Students' t-tests. P-values less than 0.05 were considered statistically significant [47,48]. Densitometric analysis of the RT-PCR and western blot results were carried out using NIH Scion Image analysis to assess the fold-change in arsenic-treated rats as compared to the control animals.

Results

Effect of arsenic on food consumption, body weight and estrous cycle

It was observed that during the entire duration of the experiment, water intake and food consumption of the control and treated rats remained unchanged. In addition, the body weights of the treated animals were not significantly different from that of the control ones. However, after 28 days of arsenic treatment, a significant decrease in the wet weight of uterus was observed in comparison to the control group (Table 2). In addition, in the control group, regular estrous cycles of 4-5 days were noted whereas, in the arsenite-treated group, a constant diestrous phase was observed after 22 ± 2 days of arsenic treatment.

Dose and time-dependent effect of arsenic on serum estradiol levels

In order to determine the effect of arsenic on serum estradiol levels in female Sprague-Dawley rats, the optimum dose responsible for the changes and the minimum time required to initiate maximum changes were resolved by ELISA of serum estradiol. Rats were treated with different doses of sodium arsenite and monitored at the end of 28 days. As depicted in Figure 1a, the serum estradiol levels reduced at a dose of 0.4 μg/ml and decreased by 4-fold in rats fed with arsenic-containing water at a dose of 4 μg/ml, as compared to control rats ($p < 0.05$). The level did not decrease further when the rats were fed arsenic-containing water at the dose of 40 μg/ml and 80 μg/ml. The minimum time required for maximum effect of arsenic was resolved to be 28 days, beyond which continuous treatment did not affect the serum estradiol levels up to a period of 56 days (Figure 1b). Hence, all subsequent experiments were performed with the optimum effective dose and time of 4 μg/ml (equivalent to 4 ppm) and 28 days, respectively.

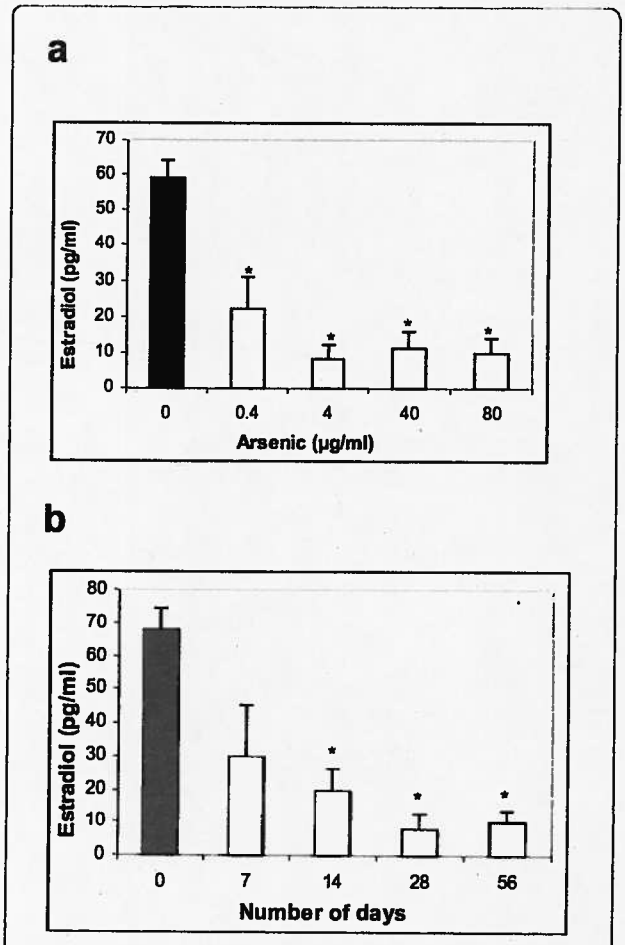


Figure 1 Serum estradiol levels of untreated and arsenic treated female Sprague Dawley rats. Rats were exposed to different doses of sodium meta-arsenite for different time periods, as mentioned in the *Methods* section. (a) Arsenic has a dose-dependent inhibitory effect on circulating estradiol levels. The dose at which maximum reduction is observed is 4 μg/ml, beyond which the level does not change significantly. Each value represented as mean ± SE, n = 3, $p < 0.05$. (b) The minimum time period required to initiate the maximum decrease in estradiol levels is 28 days. No further reduction is observed even when rats were exposed to arsenic for 56 days. Each value represented as mean ± SE, n = 3, $p < 0.05$.

Table 2: Body weight and uterine wet weight in response to 4 μg/ml arsenic treatment for 28 days in adult female rats

Treatment	Body weight (in gm)	Uterine wet weight (in mg)
Control	132 ± 1.67	146 ± 2.31
4 μg/ml $NaAsO_2$	131.6 ± 1.32	104.3 ± 1.94**

Individual weights of control and arsenic-treated rats were attained at the end of the experimental schedule. Each value represents mean ± SE, n = 5. (ANOVA followed by multiple Students' t-tests, ** $p < 0.01$, as compared with respective control).

Effect of arsenic on serum concentration of LH and FSH

LH and FSH are gonadotropins which are upstream components of estradiol signaling, and are required for the

development and quantitative maintenance of normal reproductive cycle in pubertal rats. It was observed that chronic exposure of rats to 4 μg/ml sodium arsenite for 28 days significantly decreased the serum LH and FSH concentrations (Figure 2), as detected by ELISA. Synthesis and secretion of estradiol is under the control of these gonadotropins, hence rendering them important components of sex steroid regulation. Thus, the reduction of LH and FSH may therefore be responsible for the consequent reduction in estradiol levels, as seen above.

Arsenic-induced histological changes of the uterus

Histological analysis of 4 μg/ml arsenic-treated rats showed significant alterations in the uterine morphology as compared to the untreated rats. Occulometric studies revealed rats that were exposed to arsenic showed a decrease in (i) the size and invaginations of the uterine luminal diameter, (ii) height of luminal epithelial cells, (iii) number and organization of endometrial glands, and (iv) the width of the myometrium (Table 3). The compact, tall, columnar epithelial cells lining the highly invaginated lumen of the untreated uterus (Figure 3a) were well defined as compared to the treated uterus (Figure 3b), with rounded nuclei located on a prominent basement membrane (Figure 3c). However, the height and organization of the epithelial cells were affected with arsenic treatment and revealed distortion of the cells with irregular-shaped nuclei, along with disappearance of a distinct basement membrane (Figure 3d). Additionally, the number and size of endometrial glands in the endometrial stroma were well defined in the control uterus (Figure 3e) and were significantly reduced in the treated animals (Figure 3f). Reduction in the thickness of the longitudinal muscles comprising the myometrium, as compared to the untreated uterus (Figure 3g), was also observed in the uterus of rats exposed to arsenic (Figure 3h).

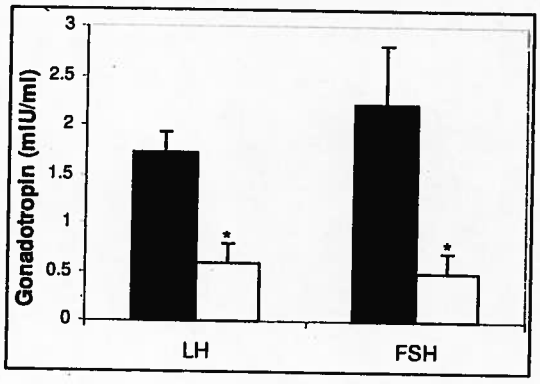


Figure 2 Serum gonadotropin levels of untreated and arsenic-treated female rats. Sprague-Dawley rats were treated with 4 μg/ml sodium meta-arsenite for 28 days, as described in the *Methods* section. The results indicate that arsenic has an inhibitory effect on serum levels of both LH and FSH (white bars), as compared to the control animals (black bars). Each value represented as mean ± SE, n = 3, $p < 0.05$.

Effect of arsenic on estrogen receptor expression

Immunohistochemical localization of estrogen receptors were detected by staining for the ERα proteins in the uteri of arsenic treated and untreated rats. Uterus from untreated rats showed presence of ERα (Figure 4a) whereas rats exposed to arsenic showed significant downregulation of ERα in the uterine endometrium (Figure 4b). Similar results were observed in the muscle layer of the control (Figure 4c) and the arsenic-treated uterus (Figure 4d). In order to further validate the effect of arsenic on the expression of estrogen receptors in the rat uterus, the expression was monitored both at the RNA transcript levels and at the protein levels. Semi-quantitative RT-PCR confirmed that the level of ERα was downregulated as a result of arsenic treatment as compared to the control uterus (Figure 5a). Western blot analyses further confirmed that concentration of the estrogen receptor protein declined by almost 2-fold as a result of exposure to arsenic (Figure 5b).

Effect of arsenic on vascular endothelial growth factor

Since arsenic is a potent environmental estrogen and responsible for downregulation of the estrogen receptor, we investigated the effects of arsenic on the estrogen signaling pathway. Concomitantly, we selected vascular endothelial growth factor (VEGF) as the estrogen-responsive gene, since it is known to respond primarily to estrogen administration in the uterine epithelium and stroma. The expression of VEGF was thus evaluated at the transcriptional and translational levels. Semi-quantitative RT-PCR revealed a 2-fold reduction of VEGF transcripts and protein in the arsenic-treated rat uterus (Figure 6), indicating that arsenic not only down regulates the estrogen receptor, but also disrupts the downstream gene expression in the rat uterus.

Effect of arsenic on the cell cycle regulatory proteins

It is well established that decreased levels of estrogen can affect the expression of the cell cycle regulators, especially those that are involved in the G1-S transition, chiefly cyclin D1 and CDK4. Concomitantly, we investigated if arsenic, which decreased the serum estradiol levels and estrogen receptor expression in female rats, would additionally alter the expression of cyclin D1 and CDK4. The results indicated significant reduction in cyclin D1 and CDK4 mRNA levels in uteri of rats exposed to arsenic, as compared to unexposed rats (Figure 7a). Concomitant downregulation in the expression of cyclin D1 and CDK4 proteins was also observed in uteri of rats exposed to arsenic treatment (Figure 7b).

Table 3: Luminal diameter, height of luminal epithelial cells, diameter of endometrial glands and width of longitudinal muscles in different experimental groups of adult female rats

Treatment	Luminal diameter (μm)[#]	Height of luminal epithelial cells (μm)[§]	Diameter of endometrial glands (μm)[§]	Width of longitudinal muscle layer (μm)[§]
Control	45.88 ± 1.87	18.44 ± 0.19	58.95 ± 3.04	75.89 ± 0.68
4 μg/ml $NaAsO_2$	10.72 ± 0.51**	5.7 ± .04***	16.61 ± 0.44**	37.69 ± 1.6**

Histological analysis was carried out for each control and arsenic-treated rat, and the uterine parameters were measured by occulometry. Each value represents mean ± SE, n = 5. (ANOVA followed by multiple Students' t-tests, ** $p < 0.01$, *** $p < 0.001$, as compared with respective controls). [#] Magnification: 10x; [§] magnification: 40x.

Discussion

In this study we report the effects of inorganic arsenic on the estrogen signaling pathway in rats, along with concomitant alterations of the uterine morphology and proliferation, to unravel the putative mechanisms behind reproductive failures associated with arsenic exposure. Rats were chronically (28 days) exposed to arsenite (4 μg/ml) in drinking water in order to establish a correlation between uterine pathology, serum estradiol and gonadotropin concentrations, and alterations in expression of the estrogen receptor and downstream components, involved with uterine tissue architecture and function, following arsenic treatment. It is evident from the results that arsenic exposure diminishes the circulating levels of both gonadotropins and estradiol. This is further supported by the fact that ovarian steroidogenesis involves enzymes regulated by the gonadotropins FSH and LH, both of which have been reported to be inhibited following exposure to arsenic [49]. Consequently, low serum levels of FSH and LH in arsenic-treated rats lead to reduction in estradiol production and thus secretion into circulation. Some reports have postulated that the decrease in gonadotropin levels may be due to an increase in plasma glucocorticoids in arsenic-treated rats, since increase in ACTH levels is known to suppress gonadotropin secretion [38].

The finding that arsenic exposure did not affect the total body weight during the exposure period, but decreased the uterine wet weight by almost 30% may be attributed to the fact that the effect of arsenic is very specific to the uterus in the tested time period. This is further supported by the fact that estradiol regulates uterine weight, and thus, low levels of estradiol lead to the specific reduction of uterine wet weight in the exposed animals. In addition, constant diestrus in arsenic-treated rats after 20 ± 2 days may also be due to low serum levels of estradiol [50-52].

In addition to altering the uterine wet weight, arsenic also leads to tissue degeneration in the uterus. The degenerative changes may be attributed to adverse effects caused by decreased serum estradiol levels, since uterine growth is primarily dependant on estradiol. The insufficient concentration of estradiol failed to maintain the normal uterine architecture and led to degeneration of the luminal epithelial cells and endometrial glands. Arsenic is known to generate reactive oxygen species and leads to oxidative damage to several components of the cell, including denaturation of proteins critical to cell functions [53-58]. Thus, degeneration of the uterine endometrial components may be associated not only with downregulated serum estradiol levels but also with increased production of reactive oxygen species following exposure to arsenic [56].

Arsenic is known to act as a potent environmental estrogen [32]. Subsequently, it was hypothesized that arsenic may mimic an estrogenic mechanism to induce lesions in the rat uterus, disrupt the estrogen signaling pathway and consequently lead to reproductive failures. Accordingly, the effect of arsenic on the expression of estrogen receptor, the estrogen responsive gene VEGF, and cell cycle regulatory proteins like CDK4 and cyclin D1 was investigated in the rat uterus. It is known that the estrogen receptor is a hormone-activated transcription factor which mediates the biological effects of estrogen in the target tissue by stimulating the expression of estrogen-regulated genes. The sensitivity of a given tissue to estrogen thus varies with the level of estrogen receptors present in it [59]. Our studies have revealed that arsenic treatment significantly downregulated the expression of ERα and its downstream element VEGF in the uterus, indicating that arsenic either suppresses the bonafide action of estradiol on the uterus by decreasing the expression of specific receptors, at both the mRNA transcript and protein levels, or acts via a parallel mechanism in the rat uterus that eventually disrupts the estrogen signaling pathway and the G1 cell cycle proteins responsible for cell proliferation. Estradiol-regulated VEGF is chiefly responsible for modulating in vivo angiogenesis in the uterus, and its downregulation by arsenic may be a primary cause for spontaneous abortions, still-births and other reproductive failures. The D-type cyclins are known to be rate-limiting for the progression through the G1 phase of the

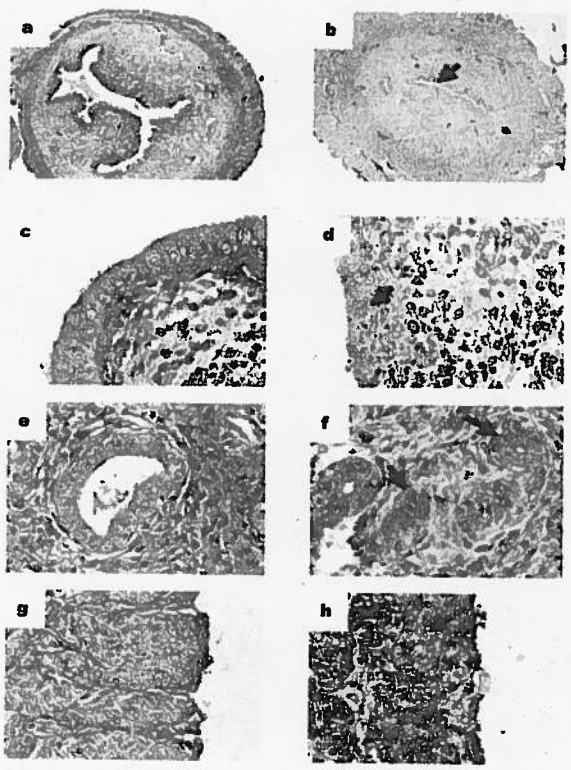


Figure 3 Alterations in tissue architecture of arsenic-treated rat uterus. Rats were treated with or without 4 μg/ml arsenic for 28 days. 3a, 3c, 3e and 3g: control sections; 3b, 3d, 3f and 3h: arsenic-treated sections. Uterine sections were subjected to double staining with hematoxylin and eosin and examined under the microscope. (3a, 3b) A significant decrease in width and invaginations of the arsenic-treated uterine lumen was observed in the treated sections (10× magnification) as compared to untreated one. (3c, 3d) Reduction in the height of luminal epithelial cells (as measured by occulometry) in the arsenic-treated sections was observed as compared to untreated one (40×). In addition, degeneration of the layer was also noted in the treated uterus. (3e, 3f) Arsenic treatment resulted in a significant reduction in the size of endometrial glands, with disappearance of the lumen and disorganization of the epithelial cells lining the glands. (3g, 3h) As compared to the control uterus, significant reduction in size and organization of the longitudinal muscle layer was observed in the arsenic-treated uterine sections.

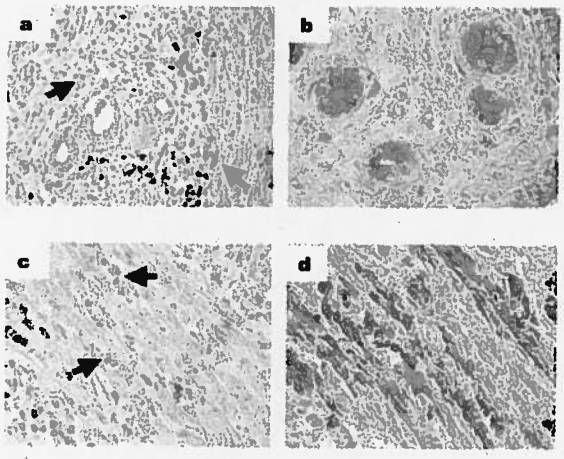


Figure 4 Immunohistochemical localization of estrogen receptor in the rat uterine sections. Rats were treated with arsenic for 28 days and tissues were stained for the presence of the estrogen receptor. Arsenic significantly downregulated the estrogen receptors in the endometrial stroma (4b) as compared to the untreated uterus (4a). In addition, the expression of estrogen receptors was also decreased in the longitudinal muscle layer (4d) in contrast to the expression in the muscle layer of the control uterus (4c).

cell cycle [60]. In fact, a strong correlation between the expression of increased levels of cyclin D1 mRNA and ER over-expression has been reported [61]. In addition, estrogens are known to increase cell proliferation by recruiting resting cells into the cell cycle, reducing the length of G1 phase and promoting entry of cells to the S phase [62]. Arsenic, on the other hand, decreased the expression of uterine estrogen receptors, and consequently suppressed cell cycle progression and reduced the proliferation-promoting effects of estradiol in the rat uterus.

Conclusions

It may thus be concluded that sodium arsenite is a non-steroidal environmental estrogen that is responsible for reducing the serum levels of gonadotropins and estradiol, which in turn lead to uterine tissue degeneration and disruption of the estrogen signaling pathway. The effects of arsenic on the uterus may occur by reducing the expression of estrogen receptors and estrogen responsive genes, and/or by generating reactive oxygen species that lead to oxidative damage of the proteins involved in the estrogen signaling pathway, that regulate the uterine structure and function. Interestingly, liver toxicity assays carried out in our laboratory did not indicate significant differences in the SGPT or SGOT levels in the experimental animals as compared to the control ones (data not shown). Hence, it may be imperative to state that level of arsenic which failed to reduce general body weight of the rats or even affect the liver toxicity enzymes, were capable of bringing about such severe detrimental changes in the uterine physiology and steroid signaling pathway. Finally, our study demonstrates that arsenic at low but chronic doses, relevant to the exposure level in different parts of the world, is a major endocrine disruptor and thus, may be

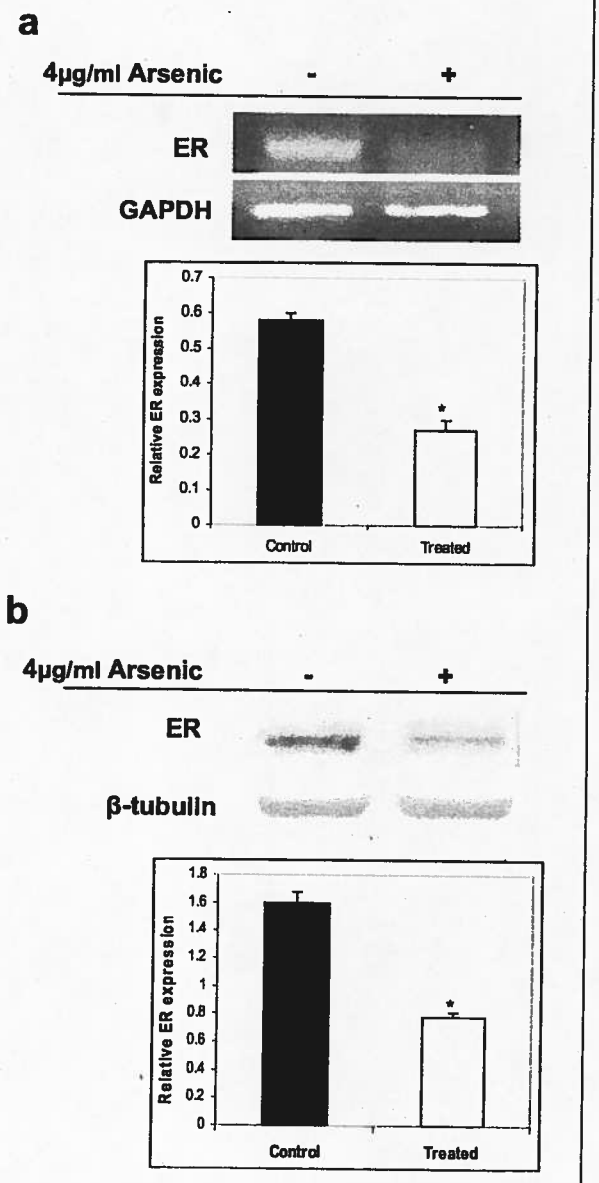


Figure 5 Effect of arsenic on estrogen receptor expression at the mRNA transcript and protein levels. Downregulation of the estrogen receptor in the arsenic-treated uterus was observed both at the mRNA transcript levels, as determined by RT-PCR (a), and at the protein levels, as determined by western blot analysis (b). The data shown is a representative of the experiments carried out in triplicate.

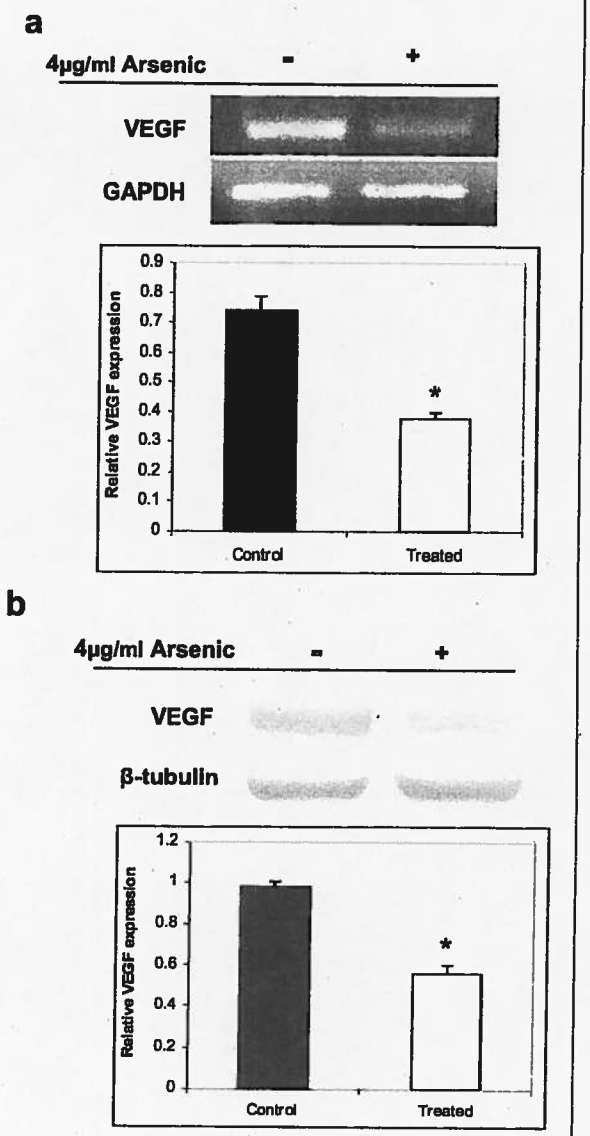
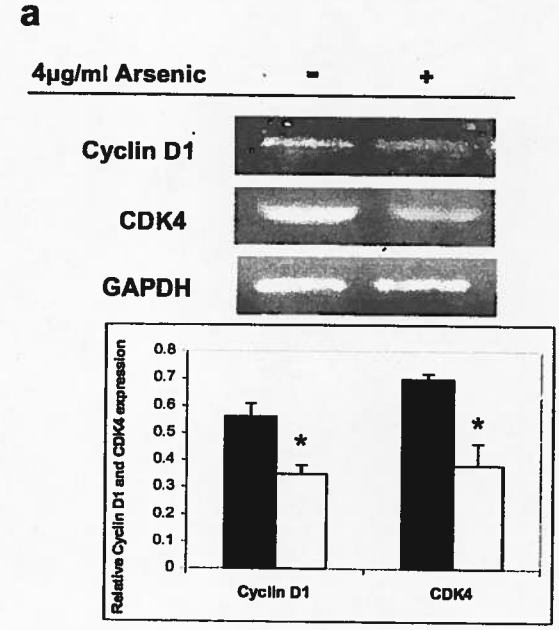


Figure 6 Arsenic downregulated VEGF expression in the rat uterus. Expression of VEGF, an estrogen-responsive gene, was observed to be downregulated both at the transcriptional (a) and translational levels (b), as determined by semi-quantitative RT-PCR and western blot analysis, respectively, in the uterus of arsenic-treated rats. The data shown is a representative of three independent experiments.

responsible for the different reproductive failures seen in women exposed to such levels of arsenic.

List of abbreviations

DEPC: Diethyl Pyro Carbonate; EDTA: Ethylene Diamine Tetra Acetic Acid; FSH: Follicle Stimulating Hormone; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; HRP: Horse Radish Peroxidase; LH: Luteinizing Hormone; MuLV: Murine Leukemia Virus; NADPH: Nicotinamide Adenine Diphosphate Nucleotide; PBS: Phosphate Buffered Saline; PBST: Phosphate Buffer Saline Tween-20; RH: Relative Humidity; RIPA: Radio Immuno Precipitation Assay; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; VEGF: Vascular Endothelial Growth Factor



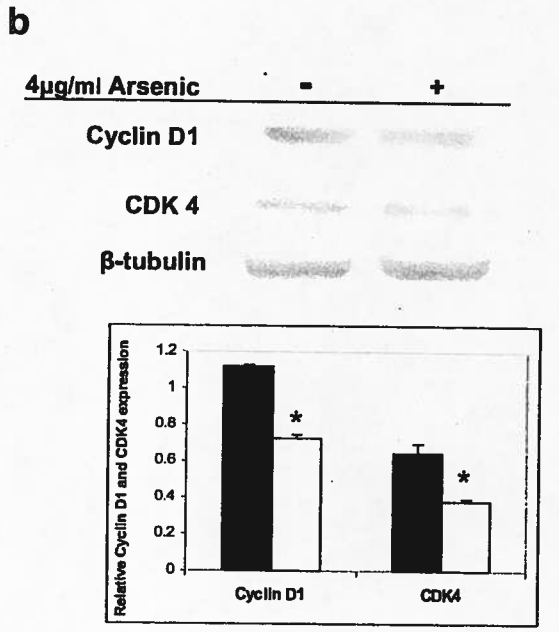


Figure 7 Regulation of G1 cell cycle proteins by arsenic in rats. Female rats were treated with arsenic at a dose of 4 µg/ml for 28 days. Cyclin D1 and CDK4 expressions were analyzed both at the mRNA levels (a) and protein levels (b), using GAPDH and β-tubulin as loading controls, respectively. It was observed that arsenic downregulated both cyclin D1 and CDK4 (white bars) in the uterus of Sprague-Dawley rats, as compared to the control animals (black bars). The data shown is a representative of the experiments carried out in triplicate.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AC carried out the treatment of animals and performed all the experiments. UC conceived the study, participated in its design, coordination, interpretation and analysis of the data. AC and UC drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Klein CB, Leszczynska J, Hickey C, Rossman TG: **Further evidence against a direct genotoxic mode of action for arsenic-induced cancer.** *Toxicol Appl Pharmacol* 2007, **222(3):**289-297.
2. Erbanova L, Novak M, Fottova D, Dousova B: **Export of arsenic from forested catchments under easing atmospheric pollution.** *Environ Sci Technol* 2008, **42(19):**7187-7192.
3. Abernathy CO, Thomas DJ, Calderon RL: **Health effects and risk assessment of arsenic.** *J Nutr* 2003, **133(5):**1536S-1538S.
4. Smith AH, Hopenhayn-Rich C, Bates MN, Goeden HM, Hertz-Piccioto I, Duggan HM, Wood R, Kosnett MJ, Smith MT: **Cancer risks from arsenic in drinking water.** *Environ Health Perspect* 1992, **97:**259-267.
5. Tapio S, Grosche B: **Arsenic in the aetiology of cancer.** *Mutat Res* 2006, **612(3):**215-246.
6. Watanabe C, Inaoka T, Matsui T, Ishigaki K, Murayama N, Ohtsuka R: **Effects of arsenic on younger generations.** *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2003, **38(1):**129-139.
7. Suk WA, Ruchirawat KM, Balakrishnan K, Berger M, Carpenter D, Damstra T, de Garbino JP, Koh D, Landrigan PJ, Makalinao I, Sly PD, Xu Y, Zheng BS: **Environmental threats to health in Southeast Asia and the Western Pacific.** *Environ Health Perspect* 2003, **111(10):**1340-1347.
8. Chiou HY, Chiou ST, Hsu YH, Chou YL, Tseng CH, Wei ML, Chen CJ: **Incidence of transitional cell carcinoma and arsenic in drinking water: a follow up study of 8102 residents in an arseniasis-endemic area in northeastern Taiwan.** *Am J Epidemiol* 2001, **153(5):**411-418.
9. Walker M, Fosbury D: **Arsenic, As (III), and tungsten in Nevada County's private water supplies.** *J Water Health* 2009, **7(2):**293-301.
10. Cai L, Liu G, Rensing C, Wang G: **Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils.** *BMC Microbiol* 2009:1-11.
11. Steinmaus C, Moore L, Hopenhayn Rich C, Biggs ML, Smith AH: **Arsenic in drinking water and bladder.** *Cancer Invest* 2000, **18(2):**174-182.
12. Karagas MR, Stukel TA, Morris JS, Tosteson TD, Weiss JE, Spencer SK, Greenberg ER: **Skin cancer risk in relation to toenail arsenic concentration in an US population based case-control study.** *Am J Epidemiol* 2001, **153(6):**559-565.
13. Liu J, Waalkes MP: **Liver is a target of arsenic carcinogenesis.** *Toxicol Sci* 2008, **105(1):**24-32.
14. Navas-Acien A, Silbergeld EK, Streeter RA, Clark JM, Burke TA, Guallar F: **Arsenic exposure and type 2 diabetes: a systematic review of the experimental and epidemiologic evidence.** *Environ Health Perspect* 2006, **114(5):**641-648.
15. Prozialeck WC, Edwards JR, Nebert DW, Woods JM, Barchowsky A, Atchison WD: **The vascular system as a target of metal toxicity.** *Toxicol Sci* 2008, **102(2):**207-218.

16. Bodwell JE, Gosse JA, Nomikos AP, Hamilton JW: **Arsenic disruption of steroid receptor gene activation: complex dose-response effects are shared by several steroid receptors.** *Chem Res Toxicol* 2006, 19(12):1619-1629.
17. Karagas MR, Stukel TA, Tosteson TD: **Assesment of cancer risk and environmental levels of arsenic in New Hampshire.** *Int J Hyg Environ Health* 2002, 205(1):85-94.
18. Chakraborti D, Rahman MM, Paul K, Chowdhury UK, Sengupta MK, Lodh D, Chanda CR, Saha KC, Mukherjee SC: **Arsenic calamity in the Indian subcontinent what lessons have been learned?** *Talanta* 2002, 58(1):3-22.
19. Arsenic health topic: [http://www.who.int/topics/arsenic/en/].
20. Kristoforovic-Ilic MJ, Bjelanovic JM, Ilic MP, Vidovic MM: **Arsenic contamination in environment in the region of Vojvodina.** *Cent Eur J Public Health* 2009, 17(3):152-157.
21. Utsunomiya S, Peters SC, Blum JD, Ewing RC: **Nanoscale mineralogy of arsenic in a region of New Hampshire with elevated as-concentrations in the ground water.** *Am Mineralogist* 2003, 88(11-12):1844-1852.
22. Smith E, Smith J, Smith L, Biswas T, Correll R, Naidu R: **Arsenic in Australian environment: an overview.** *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2003, 38(1):223-239.
23. Guo X, Fujino Y, Kaneko S, Wu K, Xia Y, Yoshimura T: **Arsenic contamination of groundwater and prevalence of arsenical dermatosis in the Hetao plain area, Inner Mongolia, China.** *Mol Cell Biochem* 2001, 222(1-2):137-140.
24. Groundwater arsenic contamination in West Bengal-India (20 years study): [http://www.soesju.org/arsenic/wb.htm].
25. Cui X, Li S, Shraim A, Kobayashi Y, Hayakawa T, Kanno S, Yamamoto M, Hirano S: **Subchronic exposure to arsenic through drinking water alters expression of cancer related genes in rat liver.** *Toxicol Pathol* 2004, 32(1):64-72.
26. Myers SI, Lobdell DT, Liu Z, Xia Y, Ren H, Li Y, Kwok RK, Mumford JL, Mendola P: **Maternal drinking water arsenic exposure and perinatal outcomes in inner Mongolia, China.** *J Epidemiol Commun Health* 2009, 64(4):325-329.
27. Waalkes MP, Liu J, Chen H, Xie Y, Achanzar WE, Zhou YS, Cheng ML, Diwan BA: **Estrogen signaling in livers of male mice with hepatocellular carcinoma induced by exposure to arsenic in utero.** *J Nat Cancer Inst* 2004, 96(6):466-474.
28. Shen J, Liu J, Xie Y, Diwan BA, Waalkes MP: **Fetal onset of aberrant gene expression relevant to pulmonary carcinogenesis in lung adenocarcinoma development induced by in utero arsenic exposure.** *Toxicol Sci* 2007, 95(2):313-320.
29. Goering PL, Aposhian HV, Mass MJ, Cebrian M, Beck BD, Waalkes MP: **The enigma of arsenic carcinogenesis: role of metabolism.** *Toxicol Sci* 1999, 49(1):5-14.
30. Barret JR: **NTP multigenerational study of environmental estrogens.** *Environ Health Perspect* 2006, 114(6):A348-349.
31. Davey JC, Bodwell JE, Gosse JA, Hamilton JW: **Arsenic as an endocrine disruptor: effects of arsenic on estrogen receptor-mediated gene expression in vivo and in cell culture.** *Toxicol Sci* 2007, 98(1):75-86.
32. Stoica A, Pentecost E, Martin MB: **Effects of arsenite on estrogen receptor-α expression and activity in MCF-7 breast cancer cells.** *Endocrinol* 2000, 114:3595-3602.
33. Hyder SM, Chiappetta C, Stancel GM: **Synthetic estrogen 17α ethinyl estradiol induces pattern of uterine gene expression similar to endogenous estrogen 17 β estradiol.** *J Pharmacol ExpTher* 1999, 290(2):740-747.
34. Gargett CE, Zaitseva M, Bucak K, Chu S, Fuller PJ, Rogers PA: **17 β estradiol up-regulates vascular endothelial growth factor receptor-2 expression in human myometrial microvascular endothelial cells: role of estrogen receptor α and β.** *J Clin Endocrinol Metab* 2002, 87(9):4341-4349.
35. Bryant W, Snowhite AE, Rice LW, Shupnik MA: **The estrogen receptor (ER) α variant delta 5 exhibits dominant positive activity on ER-regulated promoters in endometrial carcinoma cells.** *Endocrinol* 2005, 146(2):751-759.
36. Bashir S, Sharma Y, Irshad M, Nag TC, Tiwari M, Kabra M, Dogra TD: **Arsenic induced apoptosis in rat liver following repeated 60 days exposure.** *Toxicol* 2006, 217(1):63-70.
37. Waalkes MP, Ward JM, Liu J, Powell DA, Diwan BA: **Urogenital carcinogenesis in female CD1 mice induced by in utero arsenic exposure is exacerbated by postnatal diethylstilbestrol treatment.** *Cancer Res* 2006, 66(3):1337-1345.
38. Jana K, Jana S, Samanta PK: **Effects of chronic exposure to sodium arsenite on hypothalamo-pituitary-testicular activities in adult rats: possible an estrogenic mode of action.** *Rep Biol Endocrinol* 2006, 4:1-13.
39. Ratcliff WA, Carter GD, Dowsett M, Hillier SG, Middle JG, Reed MJ: **Oestradiol essays: applications and guidelines for the provision of clinical biochemistry service.** *Ann Clin Biochem* 1988, 25:466-483.
40. Ofusori DA, Caxton-Martins EA, Komolafe OO, Oluyemi KA, Adeeyo OA, Ajayi SA, Oluwayinka PO, Adelakun EA, Keji ST, Adesanya OA: **A comparative study of the ileum in rat (*Rattus norvegicus*), bat (Eidolon helvum) and pangolin (*Manis tricuspis*) as investigated using histological method.** *Int J Morphol* 2008, 26(1):137-141.
41. Bodine PV, Green J, Harris HA, Bhat RA, Stein GS, Lian JB, Komm BS: **Functional properties of a conditionally phenotypic, estrogen responsive, human osteoblast cell Line.** *J Biol Chem* 1997, 65(3):368-387.
42. Bodine PV, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS: **Estrogen receptor-α is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression.** *Endocrinol* 1998, 139(4):2048-2057.
43. Sugihara T, Wadhwa R, Kaul SC, Mitsui Y: **A novel alternatively spliced form of murine vascular endothelial growth factor, VEGF 115.** *J Biol Chem* 1998, 273(5):3033-3038.
44. Corroyer S, Maitre B, Cazals V, Clement A: **Altered regulation of G_1 cyclins in oxidant-induced growth arrest of lung alveolar epithelial cells. Accumulation of inactive cyclin E-DCK2 complexes.** *J Biol Chem* 1996, 271(41):25117-25125.
45. Kowalski AA, Graddy LG, Vale-Cruz DS, Choi I, Katzenellenbogen BS, Simmen FA, Simmen RC: **Molecular cloning of porcine estrogen receptor-β complementary DNAs and developmental expression in periimplantation embryos.** *Biol Reprod* 2002, 66(3):760-769.
46. Chatterji U, Riby JE, Taniguchi T, Bjeldanes FL, Bjeldanes LF, Firestone GL: **Indole 3-carbinol stimulates transcription of the interferon gamma receptor1 gene and augments interferon responsiveness in human breast cancer cells.** *Carcinogen* 2004, 25(7):1119-1128.
47. Cohen J, Cohen P, West SG, Aiken LS: **Applied multiple regression/ correlation analysis for the behavioral sciences.** Mahawai, NJ: Lawrence Earbaum Association Press; 2003.
48. Ferguson GA, Takane Y: **Statistical analysis in psychology and education.** Montreal: McGraw Hill Press; 2005.
49. Sarkar M, Biswas NM, Ghosh D: **Effect of sodium arsenite on testicular Δ^5-3 β, 17β-HSD activities in albino rats: dose and duration dependent responses.** *Med Sci Res* 1991, 19:789-790.
50. Chattopadhyay S, Ghosh S, Debnath J, Ghosh D: **Protection of sodium arsenite-induced ovarian toxicity by coadministration of L-Ascorbate (Vitamin C) in mature Wistar strain rat.** *Arch Environ Contam Toxicol* 2001, 41(1):83-89.
51. Chattopadhyay S, Pal Ghosh S, Ghosh D, Debnath J: **Effect of dietary co-administration of sodium selenite on sodium arsenite- induced ovarian and uterine disorders in mature albino rats.** *Toxicol Sci* 2003, 75(2):412-422.
52. Sasson R, Dantes A, Tajima K, Amsterdam A: **Novel genes modulated by FSH in normal and immortalized FSH-responsive cells: new insights into the mechanism of FSH action.** *FASEB J* 2003, 17(10):1256-1266.
53. Akram Z, Jalali S, Shami SA, Ahmad L, Batool S, Kalsoom O: **Adverse effects of arsenic exposure on uterine function and structure in female rat.** *Exp Toxicol Pathol* 2009, 62(4):451-459.
54. Kitchin KT: **Recent advances in arsenic carcinogenesis: modes of action, animal model systems and methylated arsenic metabolites.** *Toxicol Appl Pharmacol* 2001, 172(3):249-261.
55. Liu SX, Davidson MM, Tang X, Walker WF, Athar M, Ivanov V, Hei TK: **Mitochondrial damage mediates genotoxicity of arsenic in mammalian cells.** *Cancer Res* 2005, 65(8):3236-3242.
56. Colognato R, Coppede F, Ponti J, Sabbioni E, Migliore L: **Genotoxicity induced by arsenic compounds in peripheral human lymphocytes analysed by cytokinesis-block micronucleus assay.** *Mutagen* 2007, 22(4):255-261.

57. Salnikow K, Zhitkovich A: **Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic and chromium.** *Chem Res Toxicol* 2008, 21(1):28-44.
58. Liu J, Chen H, Miller DS, Saavedra JE, Keefer LK, Johnson DR, Klaassen CD, Waalkes MP: **Overexpression of glutathione S-transferase II and multidrug resistance transport proteins is associated with acquired tolerance to inorganic arsenic.** *Mol Pharmacol* 2001, 60(2):302-309.
59. Waalkes MP, Keefer LK, Diwan BA: **Induction of proliferative lesions of the uterus, testes, and liver in swiss mice given repeated injections of sodium arsenate: possible estrogenic mode of action.** *Toxicol Appl Pharmacol* 2000, 166(1):24-35.
60. Waalkes MP, Liu J, Diwan BA: **Transplacental arsenic carcinogenesis in mice.** *Toxicol Appl Pharmacol* 2007, 222(3):271-280.
61. Dickson RB, Stancel GM: **Estrogen receptor- mediated processes in normal and cancer Cells.** *J Natl Cancer Inst Monogr* 2000, 27:135-145.
62. Clarke R, Leonessa F, Welch JN, Skaar TC: **Cellular and molecular pharmacology of antiestrogen action and resistance.** *Pharmacol Rev* 2001, 53(1):25-71.

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Bohn, Brent 1123

From: Powers, Christina
Sent: Thursday, May 15, 2014 7:29 AM
To: Joca, Lauren
Cc: Powers, Christina
Subject: RE: iAs Vascular Remodeling MOA Table

Thanks Lauren! I'll take a look at this today and get back to you soon with any suggestions for revision.

Cheers,
Christy

Christy Powers
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Office of Research and Development
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From: Joca, Lauren
Sent: Wednesday, May 14, 2014 3:57 PM
To: Powers, Christina
Subject: RE: iAs Vascular Remodeling MOA Table

Hi Christy,

Included is actually the Vascular MOA write up. Please let me know of any changes I can make. Table to follow!

Best,
Lauren

From: Powers, Christina
Sent: Wednesday, May 07, 2014 1:50 PM
To: Joca, Lauren
Cc: Powers, Christina
Subject: iAs Vascular Remodeling MOA Table

Hi Lauren,

Attached is the Vascular Remodeling table with my suggestions and comments.

As always, don't hesitate to contact me with any questions. Thanks again for all of your great work on this project!

Best,
Christy

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Bohn, Brent 1128

From: Yankowski, Jessica
Sent: Wednesday, May 14, 2014 1:23 PM
To: Powers, Christina
Subject: Requested Article: Int J Cancer 2014; 135(2): 335-347
Attachments: comprehensive analyses of dna repair pathways.pdf

The article/document you requested is attached. Please reply if you have any questions or problems with the file.

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Comprehensive analyses of DNA repair pathways, smoking and bladder cancer risk in Los Angeles and Shanghai

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Tobacco smoking is a bladder cancer risk factor and a source of carcinogens that induce DNA damage to urothelial cells. Using data and samples from 988 cases and 1,004 controls enrolled in the Los Angeles County Bladder Cancer Study and the Shanghai Bladder Cancer Study, we investigated associations between bladder cancer risk and 632 tagSNPs that comprehensively capture genetic variation in 28 DNA repair genes from four DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER), non-homologous end-joining (NHEJ) and homologous recombination repair (HHR). Odds ratios (ORs) and 95% confidence intervals (CIs) for each tagSNP were corrected for multiple testing for all SNPs within each gene using pACT and for genes within each pathway and across pathways with Bonferroni. Gene and pathway summary estimates were obtained using ARTP. We observed an association between bladder cancer and *POLB* rs7832529 (BER) ($p_{ACT} = 0.003$; $p_{pathway} = 0.021$) among all, and SNPs in *XPC* (NER) and *OGG1* (BER) among Chinese men and women, respectively. The NER pathway showed an overall association with risk among Chinese males (ARTP NER $p = 0.034$). The *XRCC6* SNP rs2284082 (NHEJ), also in LD with *SREBF2*, showed an interaction with smoking (smoking status interaction $p_{gene} = 0.001$, $p_{pathway} = 0.008$, $p_{overall} = 0.034$). Our findings support a role in bladder carcinogenesis for regions that map close to or within BER (*POLB*, *OGG1*) and NER genes (*XPC*). A SNP that tags both the *XRCC6* and *SREBF2* genes strongly modifies the association between bladder cancer risk and smoking.

Key words: bladder cancer, smoking, DNA repair, *POLB*, *XRCC6*

Abbreviations: BER: Base excision repair; CI: confidence interval; df: degrees of freedom; DNA: deoxyribonucleic acid; HRR: homologous recombination repair; NHW: non-Hispanic White; NOC: N-nitroso compound; NER: nucleotide excision repair; NHEJ: non-homologous end-joining; OR: odds ratio; ROS: reactive oxygen species; SNP: single nucleotide polymorphism; tagSNP: haplotype tagging SNP

Additional Supporting Information may be found in the online version of this article.

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Urinary bladder cancer is among the 10 most common cancers worldwide, with its age standardized incidence rate varying by gender and world regions.[1] In Los Angeles County non-Hispanic white men have the highest incidence rate of bladder cancer, followed by Hispanic, African-American and Asian-American men, in spite of comparable profiles of tobacco use. Women show a similar pattern of incidence rates by race, although the overall rates are much lower than men.[2] Chinese from Shanghai have about two-third the incidence rate of bladder cancer of Chinese in Los Angeles.[3] Cigarette smoking and occupational exposure to arylamines are the main established risk factors.[4] Tobacco smoking contributes upwards of 50% of bladder cancer occurrence in men and 20% in women[5]; although more recent data suggests that in the United States the population attributable risk of smoking among men and women might now be comparable.[6] In addition to smoking and occupational exposure to arylamines,[7] use of hair dyes has been identified as a bladder cancer risk factor.[8]

Chemical carcinogens present in tobacco smoke, such as polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines and N-nitroso compounds and arylamines from other sources, can induce DNA damage in

What's new?

DNA repair plays a vital role in maintaining DNA integrity in bladder epithelial cells exposed to carcinogens from tobacco smoke. As a result, genetic variations in DNA repair genes could modify bladder cancer risk. Here, analysis of 28 genes that participate in four DNA repair pathways suggests that certain variants in base excision repair and nucleotide excision repair genes may contribute to bladder cancer formation specifically in Chinese populations. Gene-by-environment interaction analyses that included non-Hispanic whites and Chinese suggest that double strand breaks might be the most detrimental type of tobacco-induced DNA damage leading to bladder cancer.

the bladder epithelium.[9] In addition, reactive oxygen species (ROS) present in tobacco smoke,[10] and also generated as a by-product of chemical carcinogen metabolism,[11,12] can contribute to additional DNA damage. Altogether, chemical carcinogens and ROS can contribute to the accumulation of bulky adducts, single (SSB) and double strand breaks (DSB) and various forms of nucleotide base modification or loss which can lead to genomic instability. Modified or lost bases and SSBs are generally repaired through the base excision repair pathway (BER). DSBs are repaired by either the non-homologous end joining (NHEJ) or the homologous recombination repair (HRR) pathways. Bulky adducts are repaired by the nucleotide excision repair (NER) pathway.

Given the important role DNA repair pathways play in maintaining DNA integrity, it has been postulated that inter-individual genetic variation in these pathways may modify bladder cancer risk. Consistent with this hypothesis, individuals with reduced DNA repair proficiency were reported to have higher risk of developing bladder cancer.[13] Several epidemiological studies have investigated the bladder cancer associations with candidate polymorphisms in selected DNA repair genes, and a large pooled and meta-analysis of most of these studies offered support for a role of selected DNA repair variants in bladder carcinogenesis.[14] More recently, a comprehensive analysis of the NER pathway was conducted which offered further support for a role for DNA repair variants in bladder cancer risk.[15]

In this study, we report findings from an extensive pathway-based examination of 632 haplotype-tagging SNPs selected to examine common variation in coding and non-coding regions across 27 DNA repair-related genes, belonging to four DNA repair pathways: BER (*APEX1, LIG3, NEIL1, OGG1, PARP1, POLB* and *XRCC1*), NER (*ERCC1, ERCC2, ERCC4, ERCC5, LIG1, POLD1, XPA* and *XPC*), NHEJ (*DCLRE1C, LIG4, PRKDC, XRCC4, XRCC5* and *XRCC6*) and HRR (*MRE11A, NBN, RAD50, RAD51, RAD52, XRCC2* and *XRCC3*). We conducted these analyses using data from two parallel case-control studies that were similarly designed and carried out in areas of high and low bladder cancer risk: the Los Angeles Bladder Cancer Study and the Shanghai Bladder Cancer Study. We considered the potential modifying role of DNA repair SNPs on the association of gender and smoking with bladder cancer risk.

Material and Methods

Study population

Study participants were enrolled as part of two population-based case-control studies of transitional cell carcinoma of the urinary bladder conducted in Los Angeles County, California, USA and Shanghai, China. Characteristics of the Los Angeles Bladder Cancer (LABC) and Shanghai Bladder Cancer (SBC) studies have been described previously.[16,17] Briefly, the Los Angeles County Cancer Surveillance Program was used to identify cases diagnosed with histologically confirmed bladder cancer, among non-Asian cases between the ages of 25 and 68 years of age from 1987 through 1996. Using a standard procedure,[16] controls were identified among residents of the cases' neighborhoods of residence and individually (1:1) matched to cases by gender, race/ethnicity and age (±5 years). In Shanghai, the Shanghai Cancer Registry was used to identify cases diagnosed with histologically confirmed bladder cancer, residents of the city of Shanghai and between the ages of 25 and 74 years of age from 1995 to 1998. A previously described algorithm was used to randomly identify population-based controls from within the city of Shanghai,[18] who were frequency matched to bladder cancer cases by gender and five year age groups. In-person questionnaires administered to all study participants were used to collect demographic, lifestyle and medical characteristics up to the reference date, which in Los Angeles was defined for each case-control pair as two years before the case's diagnosis and in Shanghai was defined as two years prior to diagnosis for cases and two years prior to interview for controls. Mean time interval between bladder cancer diagnosis and interview was 11 months for bladder cancer cases in Los Angeles County, and 7 months for bladder cancer cases in Shanghai.[16,17] Blood specimens were collected at the time of interview. Analyses in the current study were restricted to 936 non-Hispanic Whites (NHW) from Los Angeles County (456 cases and 480 controls) and 1,056 Han Chinese from Shanghai (532 cases and 524 controls) with DNA and questionnaire data. The study was approved by Institutional Review Boards at the University of Southern California, the Shanghai Cancer Institute and the University of Pittsburgh.

Tagging SNP selection

Tagging SNPs (tagSNPs) for each DNA repair gene region were selected using Snagger[19] based on the HapMap CEPH

(Utah residents with Northern and Western European Ancestry (CEU)) population and Han Chinese in Beijing, China (CHB), population using data from HapMap release 21, July 2006. TagSNPs were selected using the following criteria: minor allele frequency (MAF) $\geq$ 5%, pairwise $r^2 \geq 0.80$ and a distance from the closest SNP greater than 60 base pairs on the Illumina platform. For each gene, the 5′-UTR- and 3′-UTR regions were extended to include SNPs ~20 kb upstream and ~10 kb downstream. In regions of no or low LD, tagSNPs with a MAF $\geq$5% at a density of ~1 per kb were selected from either HapMap or dbSNP. Finally, nonsynonymous tagSNPs and selected investigator selected SNPs were included regardless of the MAF. With the tagging approach used we were able to capture on average 95.6% (range from 83% to 100%) of genetic variation in CEU and 96.2% (range from 85% to 100%) in CHB, when considering the HapMap release 21, July 2006. This coverage is likely to be lower if we considered the more recent 1,000 Genomes as reference panel.

SNP genotyping and quality control

Peripheral blood lymphocytes were subjected to proteinase K digestion, phenol-chloroform extraction and ethanol precipitation for the purpose of DNA extraction. SNPs were genotyped on the Illumina GoldenGate BeadArray genotyping platform[20] (Illumina, Inc., San Diego, CA, USA) at the Genomics Core of the USC Norris Comprehensive Cancer Center. The Bead Studio software program was used to cluster and call genotypes according to standard Illumina protocols. In addition to Illumina QC measures, cases and controls were mixed on genotyping plates and blinded duplicate samples were included. The observed concordance for duplicate samples was >99%. Genotype data from 30 CEPH trios (Coriell Cell Repository, Camden, NJ) was also used to confirm genotyping reliability and reproducibility. TagSNPs were excluded if more than three discordant genotypes were found in comparison with genotypes from the International HapMap Project.

Further stringent criteria were applied to ensure quality genotyping data. We required that all SNPs have call rates >0.90 for the combined LABC-SBC study after eliminating SNPs which failed completely. Of the 632 SNPs, five SNPs were eliminated due to call rates of 0%. Supporting Information Table 1 describes all 627 SNPs in this study, including their minor allele frequencies among NHW and Chinese control populations. Analyses that stratified on race were restricted to SNPs with MAF $\geq$5% among Los Angeles controls (545 SNPs) or SNPs with MAF $\geq$5% among Shanghai controls (542 SNPs). Combined analyses of LABC and SBC were restricted to SNPs with MAF $\geq$5% among controls from both study sites (469 SNPs). We required all individuals had overall call rates >90% and excluded from analyses 192 individuals with overall call rates less than 90%. After excluding subjects with call rates less than 90%, we had genotyping results available for 1,800 individuals out of a total of 1,992. Individuals with genotyping data did not differ significantly from those without genotyping data for key characteristics, such as those listed in Table 1.

Deviations of observed genotype frequencies from those expected under Hardy-Weinberg equilibrium (HWE) were examined among Los Angeles and Shanghai controls separately using exact tests. The p-value when testing deviations of observed genotype frequencies from those expected under HWE was deemed significant if $p < 0.00008$ using exact tests (Bonferroni-corrected p-value; $\alpha = 0.05/627$). We did not observe evidence of deviations of observed from expected values among Los Angeles non-Hispanic white controls or Shanghai Chinese controls.

Statistical analysis

SNP main effects. In order to include all available individuals in our study, regardless of availability of 1:1 matched controls, we grouped individuals according to their reference age (<45, 45–49, 50–54, 55–59 and $\geq$60 years for Los Angeles non-Hispanic whites and <45, 45–49, 50–54, 55–59, 60–64 and $\geq$65 years for Shanghai Chinese), gender and study site and used it to group individuals in conditional logistic regression models used to estimate relative risks with odds ratios (ORs) and 95% confidence intervals (95% CI). Assuming a log-additive mode of action, we estimated per-allele ORs and 95% CI for the associations between each tagSNP and bladder cancer. Models were adjusted for smoking status (never/quit/current) in the reference year. Analyses were conducted separately by study site and jointly with adjustment for study site; we assessed for potential heterogeneity of SNP main effects across both study sites using likelihood ratio tests. Given the observed disparities in bladder cancer incidence between males and females, both in Los Angeles and Shanghai, we hypothesized that different environmental risk factors could associate with each gender. If some of these risk factors contribute to bladder carcinogenesis through the accumulation of DNA damage, we speculated that we could observe different associations between DNA repair SNPs and bladder cancer for males and females. To test this hypothesis, we assessed potential heterogeneity of SNP main effects by gender using likelihood ratios tests.

Multiple testing was conducted in a hierarchical bottom-up manner. We first corrected for multiple SNP tests within each gene region, then for multiple genes within the corresponding DNA repair pathway, and finally across all four DNA repair pathways investigated. Specifically, for each SNP within each gene region, crude p-values (p_{crude}) were corrected for multiple testing using the P_{ACT} (p-value adjusted for correlated tests) approach implemented within R.[21] We corrected for overall significance across gene regions within each pathway ($p_{pathway}$) using a Bonferroni correction of the P_{ACT} corrected p-value. Finally, we further corrected for overall statistical significance across all four investigated pathways ($p_{overall}$) using a Bonferroni correction of the pathway specific ($p_{pathway}$) p-value.

Table 1. Characteristics of non-Hispanic whites in Los Angeles County and Shanghai Chinese

	Los Angeles County			Shanghai		
	Cases $n = 456$	Controls $n = 480$		Cases $n = 532$	Controls $n = 524$	
Mean age at enrollment (SD)	56 (±7)	56 (±8)		63 (±10)	64 (±10)	
Age at enrollment (y)						
≤45	51 (11%)	62 (13%)		51 (10%)	43 (8%)	
45–49	54 (12%)	57 (12%)		30 (6%)	17 (3%)	
50–54	83 (18%)	93 (19%)		38 (7%)	25 (5%)	
55–59	138 (30%)	128 (27%)		43 (8%)	63 (12%)	
60–64	129 (28%)	104 (22%)		137 (26%)	116 (22%)	
>65	1 (0%)	35 (7%)	<0.001	233 (44%)	260 (50%)	0.016
Gender						
Male	357 (78%)	374 (78%)		421 (79%)	404 (77%)	
Female	99 (22%)	106 (22%)	0.890	111 (21%)	120 (23%)	0.424
Smoking status						
Never	83 (18%)	183 (38%)		178 (33%)	233 (44%)	
Former	173 (38%)	212 (44%)		75 (14%)	84 (16%)	
Current	200 (44%)	85 (18%)	<0.001	279 (52%)	207 (40%)	<0.001
Smoking intensity (cigarettes/day)						
Never	83 (18%)	183 (38%)		178 (33%)	233 (44%)	
<20	75 (16%)	88 (18%)		164 (31%)	155 (30%)	
≥20	298 (65%)	209 (44%)	<0.001	190 (36%)	136 (26%)	<0.001
Smoking duration (years)						
Never	83 (18%)	183 (38%)		178 (33%)	233 (45%)	
<29	162 (36%)	190 (40%)		106 (20%)	101 (19%)	
≥29	211 (46%)	107 (22%)	<0.001	248 (47%)	190 (36%)	0.001
Pack-years of smoking						
Never	83 (18%)	183 (38%)		178 (33%)	233 (44%)	
<24 pack-years	116 (25%)	151 (31%)		155 (29%)	142 (27%)	
≥24 pack-years	257 (56%)	146 (30%)	<0.001	199 (37%)	149 (28%)	0.001

Pathway analyses. In order to capture gene and pathway level effects that may not be detectable through any single SNP, we performed gene-based and pathway-based tests using the Adaptive Rank-Truncated Product (ARTP) method.[22] ARTP adaptively combines single SNP *p*-values within a gene region or a pathway to obtain a single test statistic for the gene or pathway and assesses significance of the test *via* a permutation procedure. Unlike a multiple testing procedure like P_{ACT}, which accounts for multiple SNP tests in order to properly control the type I error, ARTP combines information across SNPs within a gene or a pathway in order to increase the power to detect a gene or pathway level effect.

SNP-smoking interactions. We investigated SNP-smoking interactions considering the following smoking variables: smoking status (never, former, current), smoking intensity (never, <20, ≥20 cigarettes per day), smoking duration (never, <29, ≥29 years of smoking) and pack-years of smoking (never, <24, ≥24 pack-years). Three-level variables were generated using the median value among smoking controls as a cut point for cigarettes per day, years of smoking and pack-years. Interactions between SNPs and exposures were investigated on a multiplicative scale using conditional logistic models, assuming a log-additive mode of risk and using likelihood ratio tests that included product terms between each tagSNP and a three-level exposure variable coded with dummy variables. Tests of trend across categories of exposure were conducted by assigning median values to every tertile of exposure and modeling the categories as continuous. Interaction between SNPs and smoking status assumed that smoking status (never = 0, quit = 1, current = 2) was a categorical variable in the interaction model, while the *p*-values for trend

were calculated assuming smoking status as continuous in the interaction model.

Similar to our hierarchical approach for multiple testing correction for SNP main effects, within each gene region, crude interaction p-values for each SNP (interaction p_{crude}) were adjusted using a Bonferroni correction (P_{ACT} supports multiple tests of SNP main effects but not multiple tests of SNP by exposure interactions) that considered the number of SNPs investigated within each corresponding gene region (interaction p_{gene}). These corrected interaction p-values were further adjusted using a Bonferroni correction for the number of gene regions within each specific pathway (interaction $p_{pathway}$). Finally, these corrected interaction p-values were further adjusted using Bonferroni for pathway-wide significance (interaction $p_{overall}$), considering that a total of four pathways had been investigated. In all levels of correction, statistical significance was declared if corrected p-values were <0.05. All statistical tests conducted were two sided and all analyses were performed using Stata 11/SE (Stata Corporation, College Station, TX) and the statistical package R 2.15 (The R Project for Statistical Computing, http://www.r-project.org).

Results

Characteristics of cases and controls are summarized in Table 1. Briefly, males accounted for approximately 80% of study participants in both Los Angeles County and Shanghai. Mean age at enrollment for cases was 56 years of age in Los Angeles County and 64 years of age in Shanghai. While 44% of Shanghai cases were older than 65 years of age, less than 1% of Los Angeles cases were older than 65 years of age. Reported rates of cigarette smoking were higher among Los Angeles County cases and controls than among Shanghai cases and controls.

DNA repair SNPs and bladder cancer risk

We investigated associations between DNA repair tagSNPs and bladder cancer risk among NHW from the LABCS and Chinese from the SBCS, separately and combined. Among the 545 tagSNPs investigated among NHW in the LABC study 21 showed statistically significant associations with bladder cancer ($p_{crude} < 0.05$); however, none remained significant after within gene region correction ($p_{ACT} > 0.05$). None of these 21 tagSNPs showed statistically significant associations among Shanghai Chinese (Supporting Information Table 1).

Among the 542 tagSNPs investigated among Shanghai Chinese, 30 tagSNPs were statistically significantly associated with bladder cancer ($p_{crude} < 0.05$), and five of them remained statistically significant after multiple comparisons adjustment within gene region ($P_{ACT} < 0.05$): one in the *POLB* gene (rs7832529, OR = 1.5; 95% CI = 1.2–1.9; p_{ACT} = 0.003), one in the *POLD1* gene (rs2244095, OR = 0.8; 95%CI = 0.6–0.9; p_{ACT} = 0.025) and three in the *XPC* gene (rs2607734, OR = 1.3, 95% CI = 1.1–1.6, p_{ACT} = 0.020; rs2279017, OR = 1.3; 95%CI = 1.1–1.6, p_{ACT} = 0.024; rs2228001, OR = 1.3, 95%CI = 1.1–1.6, p_{ACT} = 0.028) (Table 2).

Among the 469 tagSNPs investigated among the LABC and SBC combined, 24 tagSNPs showed statistically significant associations with bladder cancer ($p_{crude} < 0.05$). Only 3 tagSNPs—the same ones we observed to be associated among Shanghai Chinese from the SBCS—remained statistically significant after multiple comparisons adjustment within gene region ($p_{ACT} < 0.05$): one in the *POLB* gene (rs7832529, OR = 1.5, 95% CI = 1.2–1.9, p_{ACT} = 0.003) and two in the *POLD1*gene (rs2244095, OR = 0.8, 95% CI = 0.7–0.9, p_{ACT} = 0.018; rs2546551, OR = 0.8, 95% CI = 0.7–0.9, p_{ACT} = 0.049) genes (Table 2). Of these three SNPs, only one remained statistically significant when correcting for all genes within the corresponding pathway (BER) and showed a borderline significant association when correcting for all pathways considered (*POLB* rs7832529 p_{ACT} = 0.003; $p_{pathway}$ = 0.021; $p_{overall}$ = 0.084). None of these three tagSNPs showed statistically significant heterogeneity by racial groups (NHW versus Chinese); results among Chinese and NHW were of similar magnitude and direction but were statistically significant only among Chinese. Conversely, the three tagSNPs in the *XPC* gene found to be statistically significantly associated with bladder cancer risk among Chinese showed heterogeneity by race (rs2607734 heterogeneity p = 0.041; rs2279017 heterogeneity p = 0.044; rs2228001 heterogeneity p = 0.058), with the association being restricted to Chinese.

DNA repair SNPs and smoking interactions

We conducted gene by smoking interaction analyses among NHW and Chinese combined. None of the SNPs previously identified to associate with bladder cancer risk (Table 2) were found to modify the risk of smoking on bladder cancer. *XRCC6* (rs2284082), *XPA* (rs7853179), *XRCC3* (rs709400) and *DCLRE1C* (rs1079622) were found to modify the effect of smoking across different measures of exposure, with interaction test p-values that achieved statistical significance within each gene, but not at the pathway level (Table 3). The only exception was *XRCC6* SNP rs2284082 (NHEJ pathway), which showed an interaction that achieved within gene region and within pathway and overall pathway statistical significance (Table 4). Specifically, among carriers of one (CT) or two (CC) copies of the major allele C, statistically significant trends were observed for the associations between smoking pack-years, years of smoking, cigarettes per day and smoking status, with greater strengths of association for CC carriers than CT carriers. Instead, among carriers of two copies of the minor allele T (TT), non-statistically significant positive trends, with reduced estimates, were observed (Table 4). For all smoking variables considered, except cigarettes per day, tests of interaction remained statistically significant after correction for multiple testing at the gene and pathway levels (Smoking pack-years interaction p_{gene} = 0.003, $p_{pathway}$ = 0.020; years of smoking interaction p_{gene} = 0.008, $p_{pathway}$ =

Table 2. SNPs associated with bladder cancer risk in the Los Angeles-Shanghai study

Pathway	Gene	tagSNP	MAF	Ca	Co	OR[1]	LCI	UCI	p_{crude}	p_{ACT}	$p_{pathway}$	$p_{overall}$	p_{Het}
NHW (LABC)													
BER	*POLB*	rs7832529	0.05	351	405	1.4	0.9	2.1	0.186	0.662	1.000	1.000	
NER	*POLD1*	rs2244095	0.11	353	407	0.9	0.6	1.2	0.473	0.884	1.000	1.000	
NER	*POLD1*	rs2546551	0.44	353	404	0.8	0.7	1.0	0.066	0.354	1.000	1.000	
NER	*XPC*	rs2607734	0.43	355	409	1.0	0.8	1.3	0.857	1.000	1.000	1.000	
NER	*XPC*	rs2279017	0.43	354	408	1.0	0.8	1.3	0.873	1.000	1.000	1.000	
NER	*XPC*	rs2228001	0.43	352	409	1.0	0.8	1.3	0.773	1.000	1.000	1.000	
Chinese (SBC)													
BER	*POLB*	rs7832529	0.12	509	518	1.5	1.2	2.0	0.001	0.009	0.060	0.239	
NER	*POLD1*	rs2244095	0.35	513	514	0.8	0.6	0.9	0.004	0.025	0.173	0.693	
NER	*POLD1*	rs2546551	0.16	512	512	0.8	0.6	1.0	0.049	0.219	1.000	1.000	
NER	*XPC*	rs2607734	0.36	514	520	1.3	1.1	1.6	0.002	0.020	0.141	0.562	
NER	*XPC*	rs2279017	0.36	510	520	1.3	1.1	1.6	0.003	0.024	0.168	0.670	
NER	*XPC*	rs2228001	0.36	513	521	1.3	1.1	1.6	0.004	0.028	0.197	0.788	
NHW & Chinese (LABC & SBC)													
BER	*POLB*	rs7832529		860	923	1.5	1.2	1.9	<0.001	0.003	0.021	0.084	0.564
NER	*POLD1*	rs2244095		866	921	0.8	0.7	0.9	0.003	0.018	0.125	0.500	0.350
NER	*POLD1*	rs2546551		865	916	0.8	0.7	0.9	0.009	0.049	0.342	1.000	0.676
NER	*XPC*	rs2607734		869	929	1.2	1.0	1.4	0.015	0.095	0.667	1.000	0.041
NER	*XPC*	rs2279017		864	928	1.2	1.0	1.4	0.018	0.111	0.778	1.000	0.044
NER	*XPC*	rs2228001		865	930	1.2	1.0	1.4	0.016	0.101	0.709	1.000	0.058

[1]Per allele ORs and 95% CIs estimated from conditional logistic regression models assuming a log-additive mode of risk and adjusting for smoking status in reference year

LCI = 95% lower confidence interval; UCI = 95% upper confidence interval; p_{crude} = unadjusted for multiple testing p-value; p_{ACT} = p-value corrected for multiple testing within gene region; $p_{pathway}$ = p-value corrected for multiple testing within gene region and within pathway; $p_{overall}$ = p-value corrected for testing across all SNPs and pathways; p-Het = LRT p-value from test of heterogeneity.

Table 3. DNA repair SNPs × smoking interactions among NHW from Los Angeles County & Shanghai Chinese

Exposure	# SNPs with interaction p_{crude} < 0.05	Pathway	Gene	SNP	Interaction p_{crude}	Interaction p_{gene}	Interaction $p_{pathway}$	Interaction $p_{overall}$
Years of smoking	29	NHEJ	*XRCC6*	rs2284082	0.001	0.008	0.046	0.185
		NER	*XPA*	rs7853179	0.002	0.023	0.164	0.656
		HR	*XRCC3*	rs709400	0.003	0.036	0.250	0.999
Pack-years of smoking	26	NHEJ	*XRCC6*	rs2284082	<0.001	0.003	0.020	0.079
		NHEJ	*DCLRE1C*	rs10796227	0.002	0.033	0.199	0.794
Cigarettes per day	8	NHEJ	*XRCC6*	rs2284082	0.015	0.093	0.556	1.000
Smoking Status	35	NHEJ	*XRCC6*	rs2284082	<0.001	0.001	0.008	0.034
		HR	*XRCC3*	rs709400	0.002	0.025	0.177	0.706
		NER	*XPA*	rs7853179	0.003	0.048	0.338	1.000
		NHEJ	*DCLRE1C*	rs10796227	0.004	0.050	0.302	1.000

Table 4. XRCC6 rs2284082 x smoking interactions and bladder cancer risk among NHW from Los Angeles County & Shanghai Chinese

	Cases/Controls			CC				CT				TT				Interaction p-values	
Smoking variables	**CC**	**CT**	**TT**	**OR**	**LCI**	**UCI**	**p-value**	**OR**	**LCI**	**UCI**	**p-value**	**OR**	**LCI**	**UCI**	**p-value**		
Smoking pack-yrs																	
Never	67/144	111/174	54/57	1.0				1.0				1.0				p_{crude}	0.001
<24 pack-years	84/112	105/99	45/45	1.9	1.3	2.8	0.001	1.6	1.2	2.2	<0.001	1.4	0.9	2.3	0.151	p_{gene}	0.003
≥24 pack-years	154/91	173/129	45/42	4.3	3.0	6.2	<0.001	2.4	1.9	3.2	<0.001	1.4	0.9	2.2	0.193	$p_{pathway}$	0.020
p for trend							<0.001				<0.001				0.288	$p_{overall}$	0.079
Years of smoking																	
Never	67/144	111/174	54/57	1.0				1.0				1.0				p_{crude}	0.001
<29	90/109	95/104	38/36	1.8	1.2	2.7	0.003	1.5	1.1	2.0	0.007	1.2	0.7	2.1	0.417	p_{gene}	0.008
≥29	148/94	183/124	52/51	4.4	3.0	6.4	<0.001	2.6	2.0	3.4	<0.001	1.5	1.0	2.4	0.077	$p_{pathway}$	0.046
p for trend							<0.001				<0.001				0.077	$p_{overall}$	0.185
Cigarettes per day																	
Never	67/144	111/174	1.0				1.0					1.0				p_{crude}	0.015
<20	81/84	95/94	38/41	2.3	1.5	3.4	<0.001	1.7	1.3	2.2	<0.001	1.2	0.8	2.0	0.405	p_{gene}	0.093
≥20	157/119	183/134	52/46	3.5	2.4	5.0	<0.001	2.3	1.8	3.0	<0.001	1.5	1.0	2.4	0.069	$p_{pathway}$	0.556
p for trend							<0.001				<0.001				0.068	$p_{overall}$	1.000
Smoking Status																	
Never	67/144	111/174	54/57	1.0				1.0				1.0				p_{crude}	<0.001
Former	78/118	87/100	30/31	1.6	1.1	2.4	0.022	1.4	1.1	1.9	0.021	1.3	0.7	2.1	0.394	p_{gene}	0.001
Current	160/85	191/128	60/56	4.7	3.3	6.9	<0.001	2.6	2.0	3.4	<0.001	1.4	0.9	2.3	0.123	$p_{pathway}$	0.008
p for trend							<0.001				<0.001				0.125	$p_{overall}$	0.032

LCI = 95% lower confidence interval; UCI = 95% upper confidence interval; p_{crude} = unadjusted for multiple testing *p*-value; p_{gene} = *p*-value corrected for multiple testing within gene region; $p_{pathway}$ = *p*-value corrected for multiple testing within gene region and within pathway; $p_{overall}$ = *p*-value corrected for testing across all SNPs and pathways.

0.046; smoking status interaction $p_{gene} = 0.001$, $p_{pathway} = 0.008$) (Table 4). Test of interaction for smoking status also remained statistically significant when further correcting for the total number of DNA repair pathways investigated (smoking pack-years interaction $p_{overall} = 0.032$) (Table 4).

DNA repair SNPs by gender interactions

To explore possible heterogeneity of the SNP-bladder cancer associations, we conducted stratified analysis by gender among NHW, Chinese and among both sites combined (Table 5). Among NHW males but not NHW females, we observed inverse associations for three linked *LIG1* SNPs (rs2007183, rs20579 and rs3730912) with bladder cancer that were statistically significant after within-gene-region correction ($p_{ACT} < 0.05$) and showed evidence of heterogeneity by gender ($p_{heterogeneity} < 0.05$) (Table 5).

Among Chinese, we observed three tagSNPs in the *OGG1* gene that showed evidence of statistically significant heterogeneity by gender. These three SNPs were inversely associated with bladder cancer risk only among females, and the associations remained statistically significant after within-gene corrections, and for one of them remained significant after pathway correction as well (rs6809452, OR = 0.5; 95% CI = 0.3–0.8, $p_{ACT} = 0.007$, $p_{pathway} = 0.046$; rs1052133, OR = 0.6, 95% CI = 0.4–0.8, $p_{ACT} = 0.026$; rs2072668, OR = 0.6, 95% CI = 0.4–0.9, $p_{ACT} = 0.049$). Similar estimates were observed among NHW females and among NHW and Chinese females combined, but estimates did not reach statistical significance (data not shown). We also observed that the previously observed associations of the *POLB* tagSNP (rs7832529) and the 3 *XPC* tagSNPs (rs26077734, rs2228001 and rs2279017) with bladder cancer risk among all Chinese individuals combined, plus an additional new *XPC* tagSNP (rs2305843), seemed restricted to males, but tests of heterogeneity were not statistically significant (Table 5).

Similarly, among males in the combined study (NHW and Chinese), three *XPC* tagSNPs (rs2305843 rs2607734 and rs2228001) were statistically significantly associated with bladder cancer risk. In addition, the previously observed association between the *POLD1* tagSNPs (rs2546651 and rs2244095) and bladder cancer risk among both races combined seemed restricted to males. However, for neither of these tagSNPs were tests of heterogeneity by gender statistically significant (Table 5).

Pathway analyses

We used the ARTP approach to obtain a summary *p*-value for the association of each gene and pathway considered in the study with bladder cancer risk (Table 6). Among NHW, only *LIG1* (NER pathway) achieved gene-wide statistical significance among males. Instead, among Chinese, six genes appeared associated with susceptibility to bladder cancer achieving ARTP gene-wide significance, with four of them showing heterogeneity by gender: *OGG1* (Chinese females $p_{ARTP\ gene} = 0.015$), *POLB* (All Chinese $p_{ARTP\ gene} = 0.010$, Chinese males $p_{ARTP\ gene} = 0.048$), *RAD50* (All Chinese $p_{ARTP\ gene} = 0.034$, Chinese males $p_{ARTP\ gene} = 0.023$), *POLD1* (All Chinese $p_{ARTP\ gene} = 0.021$), *XPC* (All Chinese $p_{ARTP\ gene} = 0.017$, Chinese males $p_{ARTP\ gene} = 0.003$) and finally *XRCC6* (All Chinese $p_{ARTP\ gene} = 0.010$, Chinese females $p_{ARTP\ gene} = 0.043$). Three of these genes showed ARTP gene-wide significance when all NHW and Chinese combined: *POLB* (Chinese & NHW $p_{ARTP\ gene} = 0.013$), *RAD50* (Chinese & NHW $p_{ARTP\ gene} = 0.048$), *POLD1* (Chinese & NHW $p_{ARTP\ gene} = 0.013$) and *XPC* (Chinese & NHW $p_{ARTP\ gene} = 0.045$) (Table 6). When considering overall pathway associations, we only observed an association of pathway-wide significance for the NER pathway among Chinese males ($p_{ARTP\ pathway} = 0.034$), and we observed a pathway-wide ARTP *p*-value of borderline significance when considering all Chinese combined ($p_{ARTP\ pathway} = 0.068$) (Table 6).

Discussion

In this study we investigated the association between a comprehensive SNP panel that captured genetic variation in genes that play key roles in four different DNA repair pathways and bladder cancer risk. Our most consistent and key findings were an association between *POLB* rs7832529 and bladder cancer risk, predominantly among Chinese, an association between *OGG1* rs6809452 and bladder cancer risk among Chinese women only and an association between *XPC* rs2607734 and bladder cancer risk among Chinese men only. POLB and OGG1 play key roles in the BER pathway and XPC participates in the NER pathway. Analyses that summarized the effects of all SNPs within each gene, obtained using the ARTP approach for both genders combined confirmed a role for *POLB* in bladder cancer risk among Chinese and also indicated associations between *RAD50* (HRR pathway), *POLD1* (NER pathway), *XPC* (NER pathway), *LIG1* (NER pathway), *OGG1* (BER pathway) and *XRCC6* (NHEJ pathway). However, when considering estimates that summarized the effect of all genes within each of the four pathways, we observed only a statistically significant association for the NER pathway among Chinese males and a borderline statistically significant one among all Chinese combined. When considering cigarette smoking variables we found consistent evidence that the *XRCC6* rs2284082 SNP (NHEJ pathway) modified the effect of smoking. Estimates of interaction for this SNP remained statistically significant after correction for multiple testing within each gene, within the NHEJ pathway and across all four pathways. None of the genes in the other three pathways showed strong evidence of effect modification by smoking. Altogether, these findings suggest that among Chinese, particularly men, there are bladder cancer risk factors, other than smoking, that elicit the BER and NER pathways and may play key roles in bladder cancer formation. Alternatively, they suggest that presence of these genetic variants, may predispose individuals to developing bladder cancer, independently of environmental exposures, perhaps due to loss over time of DNA repair proficiency and inability to repair DNA damage that may accumulate with age.

Table 5. SNPs associated with bladder cancer risk, among males and females, in the Los Angeles-Shanghai study

			Males								Females									
Pathway	Gene	SNP	CA	CO	OR[1]	LCI	UCI	p_{crude}	p_{ACT}	$p_{pathway}$	$p_{overall}$	CA	CO	OR[1]	LCI	UCI	p_{crude}	p_{ACT}	$p_{pathway}$	$p_{overall}$	LRp
NHW																					
NER	*LIG1*	rs2007183	277	322	0.6	0.4	0.9	0.005	0.041	0.288	1.000	76	86	1.7	0.8	3.8	0.165	0.609	1.000	1.000	0.013
NER	*LIG1*	rs20579	279	323	0.6	0.4	0.9	0.006	0.044	0.308	1.000	76	86	1.9	0.9	4.0	0.113	0.487	1.000	1.000	0.008
NER	*LIG1*	rs3730912	279	323	0.6	0.4	0.9	0.008	0.055	0.387	1.000	76	86	1.7	0.7	4.0	0.230	0.656	1.000	1.000	0.027
Chinese																					
NER	*XPC*	rs2607734	407	400	1.5	1.2	1.8	0.001	0.005	0.032	0.127	107	120	1.0	0.7	1.5	0.987	1.000	1.000	1.000	0.092
NER	*XPC*	rs2228001	406	402	1.4	1.2	1.8	0.001	0.008	0.055	0.219	107	119	1.0	0.7	1.5	0.991	1.000	1.000	1.000	0.107
NER	*XPC*	rs2279017	403	401	1.4	1.2	1.8	0.001	0.008	0.056	0.223	107	119	1.0	0.7	1.5	0.902	1.000	1.000	1.000	0.139
NER	*XPC*	rs2305843	406	401	1.4	1.1	1.7	0.004	0.033	0.227	0.909	107	120	1.0	0.7	1.4	0.764	1.000	1.000	1.000	0.098
BER	*POLB*	rs7832529	403	398	1.5	1.1	2.0	0.009	0.057	0.400	1.000	106	120	1.8	1.0	3.2	0.043	0.237	1.000	1.000	0.524
BER	*OGG1*	rs6809452	407	402	0.9	0.7	1.1	0.324	0.720	1.000	1.000	107	120	0.5	0.3	0.8	0.001	0.007	0.046	0.184	0.011
BER	*OGG1*	rs1052133	404	402	0.9	0.8	1.1	0.443	0.773	1.000	1.000	107	119	0.6	0.4	0.8	0.004	0.026	0.179	0.717	0.025
BER	*OGG1*	rs2072668	405	402	0.9	0.8	1.2	0.518	1.000	1.000	1.000	107	118	0.6	0.4	0.9	0.008	0.049	0.342	1.000	0.038
NHW & Chinese																					
BER	*POLB*	rs7832529	678	719	1.4	1.1	1.8	0.015	0.076	0.534	1.000	182	204	2.1	1.3	3.4	0.004	0.024	0.170	0.679	0.145
NER	*XPC*	rs2305843	683	715	1.3	1.1	1.6	0.004	0.030	0.211	0.845	183	204	1.0	0.7	1.4	0.948	1.000	1.000	1.000	0.131
NER	*XPC*	rs2607734	686	723	1.3	1.1	1.5	0.006	0.040	0.282	1.000	183	206	1.0	0.7	1.3	0.984	1.000	1.000	1.000	0.190
NER	*XPC*	rs2228001	683	725	1.3	1.1	1.5	0.006	0.041	0.290	1.000	182	205	1.0	0.7	1.3	0.981	1.000	1.000	1.000	0.178
NER	*POLD1*	rs2546551	683	714	0.8	0.6	0.9	0.007	0.045	0.314	1.000	182	202	0.9	0.6	1.3	0.601	1.000	1.000	1.000	0.419
NER	*POLD1*	rs2244095	683	718	0.8	0.6	0.9	0.009	0.050	0.347	1.000	183	203	0.8	0.5	1.1	0.135	0.390	1.000	1.000	0.932

[1]Per allele ORs and 95% CIs estimated from conditional logistic regression models assuming a log-additive mode of risk and adjusting for smoking status in reference year
LCI = 95% lower confidence interval; UCI = 95% upper confidence interval; p_{crude} = unadjusted for multiple testing p-value; p_{ACT} = p-value corrected for multiple testing within gene region; $p_{pathway}$ = p-value corrected for multiple testing within gene region and within pathway; $p_{overall}$ = p-value corrected for testing across all SNPs and pathways; LRp = LRT p-value from test of heterogeneity

Table 6. Gene- and pathway-level summary p-values from ARTP pathway analyses in LABCS and SBCS

		ARTP Gene *p*-value							ARTP Pathway *p*-value						
		NHW			Chinese				NHW			Chinese			
Pathway	Gene/ Region	All	Females	Males	All	Females	Males	Combined All	All	Females	Males	All	Females	Males	Combined All
BER									0.746	0.443	0.4845	0.356	0.143	0.329	0.724
	APEX1	0.926	0.932	0.898	0.831	0.920	0.791	0.926							
	LIG3	0.431	0.061	0.904	0.834	0.891	0.767	0.474							
	NEIL1	0.633	0.727	0.712	0.881	0.922	0.724	0.894							
	OGG1	0.674	0.678	0.812	0.225	0.015	0.556	0.486							
	PARP1	0.170	0.650	0.066	0.895	0.085	0.761	0.628							
	POLB	0.416	0.248	0.980	0.010	0.262	0.048	0.013							
	XRCC1	0.736	0.252	0.828	0.209	0.887	0.116	0.694							
HRR									0.554	0.587	0.784	0.510	0.772	0.219	0.676
	MRE11A	0.272	0.239	0.285	0.805	0.589	0.849	0.566							
	NBN	0.380	0.310	0.366	0.471	0.718	0.651	0.795							
	RAD50	0.591	0.189	0.871	0.034	0.824	0.023	0.048							
	RAD51	0.214	0.923	0.196	0.930	0.965	0.970	0.402							
	RAD52	0.967	0.488	0.959	0.821	0.622	0.946	0.935							
	XRCC2	0.154	0.267	0.466	0.779	0.155	0.978	0.276							
	XRCC3	0.384	0.872	0.877	0.321	0.395	0.255	0.664							
NER									0.649	0.672	0.234	0.068	0.293	0.034	0.107
	ERCC1-ERCC2		0.682	0.824	0.740	0.133	0.161	0.193	0.087						
	ERCC4	0.312	0.2105	0.918	0.069	0.160	0.267	0.487							
	ERCC5	0.177	0.7625	0.181	0.332	0.916	0.462	0.360							
	LIG1	0.139	0.3745	0.025	0.716	0.302	0.795	0.942							
	POLD1	0.430	0.4705	0.315	0.021	0.057	0.105	0.013							
	XPA	0.956	0.1245	0.985	0.915	0.491	0.806	0.886							
	XPC	0.563	0.9705	0.440	0.017	0.941	0.003	0.045							
NHEJ									0.598	0.182	0.698	0.236	0.206	0.774	0.397
	DCLRE1C	0.975	0.190	0.7845	0.728	0.216	0.942	0.717							
	LIG4	0.715	0.241	0.841	0.908	0.711	0.935	0.841							
	PRKDC	0.587	0.352	0.7015	0.789	0.298	0.840	0.540							
	XRCC4	0.104	0.125	0.140	0.174	0.247	0.315	0.129							
	XRCC5	0.768	0.629	0.678	0.292	0.333	0.280	0.595							
	XRCC6	0.821	0.076	0.808	0.038	0.043	0.284	0.458							

Finally, our findings support a role for the NHEJ pathway in smoking-induced bladder cancer risk, suggesting that among all types of damage induced by tobacco carcinogens, double strand breaks seem to be the ones more detrimental for cancer risk. In support of this, two other NHEJ genes (*DCLRE1C* and *XRCC3*) were also found to modify the effect of smoking, although findings were not as significant as for *XRCC6*.

The number of variants and genes investigated in DNA repair pathways in association with bladder cancer risk has been limited. In collaboration with the International Consortium of Bladder Cancer Studies, we previously published a meta-analysis and pooled analyses of 10 common variants in seven genes and reported that three SNPs (*ERCC2* rs1799793, *NBN* rs1805794 and *XPC* rs2228000) were associated with a modest increase in bladder cancer risk.[14] GWAS, meta-analysis of GWAS and pathway-based analysis of GWAS have identified multiple loci associated with bladder cancer susceptibility in subjects of European ancestry.[23–29] Whereas several SNPs located in carcinogen metabolism enzyme coding genes have achieved genome-wide significance, no SNPs located in DNA repair genes have achieved genome-wide significance to date. We summarize below what is known about

the genetic regions for which we found stronger evidence of an association with bladder cancer risk (*XPC, POLB, OGG1* and *POLD)* and evidence of interaction with smoking (*XRCC6).*

Our pathway-based analyses point to the NER pathway as relevant for bladder cancer risk. Associations between SNPs in the *XPC* and *POLD1* genes among Chinese seemed to be responsible for the overall observed association with this pathway. NER is involved in the repair of bulky DNA adducts, such as those induced by tobacco smoke carcinogens.[30] The xeroderma pigmentosum complementation group C gene (*XPC*) (HGNC 12816) is located on chromosome 3p25. XPC detects and binds to DNA adducts and initiates recruitment of other NER pathway proteins at the site of damage.[31,32] Our individual SNP analyses and overall gene analyses suggested an association between bladder cancer risk and *XPC*. Pooled analyses of most available epidemiological studies with data on selected *XPC* polymorphisms, including ours, showed an association for *XPC* rs2228000 with bladder cancer risk among NHW and no association with SNP rs2228001.[14] In this study, we could not replicate the association with rs2228000 among NHW or Chinese; however, we report a statistically significant association between *XPC* rs2228001 and bladder cancer risk among Chinese males.[14] The functional relevance/biological mechanism of the variant is unknown. There are two 3′UTR SNPs nearby that have been reported to affect XPC protein expression: rs2470352 and rs2470458[33]; however, neither of these SNPs are in LD with rs2228001.

Our individual SNP analyses and overall gene analyses also indicated an association between *POLD1* and bladder cancer risk, which seem stronger among men. The polymerase (DNA directed), delta 1, catalytic subunit gene (*POLD*) (HGNC: 9175) is located on chromosome 19q13 and encodes the catalytic and proofreading subunit of Pol δ, which has polymerase and 3′-exonuclease activity.[34] We report associations with bladder cancer risk for two SNPs: rs2546551, an intronic SNP and rs2244095 SNP, which is 3′-downstream of *POLD*, within the Spi-B transcription factor (Spi-1/PU.1 related) gene (*SPIB*) (HGNC: 11242). Both SNPs are unlinked among Chinese and among NHW (HapMap CHBJPT $r^2 = 0.22$, $D' = 0.93$; CEU $r^2 = 0.12$, $D' = 1.00$). These SNPs are not linked with previously SNPs investigated in relation to bladder cancer risk, for which no associations were reported.[35–37]

We found that SNP rs7832529 in *POLB* associated with bladder cancer risk, mostly among Chinese. Summary estimates at the gene level using ARTP supported this finding. The polymerase (DNA directed) beta gene (*POLB*) (HGNC: 9174) is located on chromosome 8p11 and encodes a DNA polymerase involved in short patch and long patch BER.[38] Bladder cancer tumors and cell lines frequently encounter deletions in chromosomal region 8p, with 8p11-12 being one of the affected regions.[39] Located 3′-downstream from *POLB*, SNP rs7832529 is actually located within the solute carrier family 20 (phosphate transporter), member 2 gene (*SLC20A2*) (HGNC: 10947). To our knowledge, *SLC20A2* has not been linked with bladder cancer. Several other *POLB* SNPs have been reported to be associated with bladder cancer risk among Caucasians, but neither are in LD with rs7832529.[37,40] It remains to be determined whether rs7832529 is tagging a causal SNP in *POLB* or *SLC20A2*.

We also report that three *OGG1* SNPs (rs2072668, rs6809452 and rs1052133) were inversely associated with bladder cancer risk among Chinese females, with a stronger association for rs6809452. The 8-oxoG DNA glycosylase1 gene (*OGG1*) (HGNC: 12816) is located on chromosome 3p26.[41] The OGG1 protein participates in the removal of 8-oxoguanine (8-oxo-G) DNA damage that can result from ROS exposure. The intronic *OGG1* rs2072668 and rs6809452 SNPs were in strong LD with the non-synonymous and putative functional *OGG1* Ser326Cys SNP (rs1052133) (HapMap CHBJPT $r^2 = 0.98$, $D' = 1.00$ for rs2072668 and $r^2 = 0.88$, $D' = 1.00$ for rs6809452). SNP rs6809452, for which we found the strongest association, is actually an intronic SNP within the transcriptional adapter 3-like gene *(TADA3L).* The *OGG1* Ser326Cys rs1052133 Cys allele has been reported to code for a protein with decreased ability to repair oxidative DNA damage.[42–46] A meta-analysis of various cancers reported that Ser326Cys was significantly associated with overall cancer risk and lung cancer risk but was not associated with bladder cancer risk.[47] Three epidemiological studies have reported associations between this SNP and bladder cancer risk among Caucasians, with stronger associations among smokers.[35–37]

Finally, we observed strong evidence that one SNP in the *XRCC6* gene, rs22284082, modified the effect of cigarette smoking. We found that among carriers of one or two copies of the C allele (major allele) there was a stronger and more significant association with tobacco smoking that among carriers of two copies of the T allele. The X-ray repair complementing defective repair in Chinese hamster cells 6 gene (*XRCC6*) (HGNC: 4055) is on chromosome region 22q13. SNP rs22284082 is located 3′-downstream from *XRCC6* and it maps to the sterol regulatory element binding transcription factor 2 gene (*SREBF2*) (HGNC: 11290), in intron 1. *SRBF2* encodes a transcription factor SREBP-2, a basic helix-loop-helix-leucine zipper protein that can stimulate transcription of sterol regulated genes and monitor lipid homeostasis.[48] In addition, SREBP-2 can also regulate autophagy related genes in times of nutrient depletion.[49] *SREBF2* has not been investigated in relation with bladder cancer; however, it has been reported to be involved in the loss of sterol feedback regulation in cancer cells.[50] It remains to be determined if the interaction with smoking we see for this SNP is capturing an effect of a causal SNP in *XRCC6* or *SREBF2*.

Our study had several strengths. Among them, was the use of two population-based case-control studies conducted in parallel in two world regions with contrasting bladder cancer incidence, using comparable instruments to assess

smoking exposure. Another one is the use of a comprehensive tagSNP approach that captured 85–100% genetic variation in genes that play key roles in four major DNA repair pathways, with appropriate consideration of multiple testing. Although we recognize that our tagSNP selection was done before the release of the 1,000 genomes project, which includes rare variants. Therefore, compared to this reference database, our overall genetic coverage would be lower. Finally, given that most studies on DNA repair susceptibility genes and bladder cancer have been conducting among NHW, our study contributes novel data about genetic risk factors among Chinese. Among the limitations of our study we include the fact that not all DNA repair genes from each pathway were captured, albeit all those that play essential roles were included and the fact that we were underpowered to explore higher order interactions between genes and exposures. Lastly, in spite of our approaches for multiple testing correction, we cannot discard the possibility that some of our findings might be false positives. Replication in other studies will help confirm our findings.

In conclusion, we found support that two regions that map close to or within BER genes (*POLB, OGG1*) and one region in an NER gene (*XPC*) are associated with bladder cancer risk, primarily among Chinese. Given that these associations were not modified by smoking, they suggest that there are other environmental factors that elicit the BER and NER pathways and might be relevant bladder cancer risk factors. We also find evidence that one SNP that tags both the *XRCC6* and *SREBF2* genes strongly modifies the association between bladder cancer risk and tobacco smoke. Given the role XRCC6 plays in the NHEJ pathway, this finding suggests that tobacco smoking may induce bladder cancer through the formation of double strand breaks. Further investigation in independent study populations will help confirm these findings and guide future studies to identify the causal variants responsible for these associations and all the relevant exposures that elicit the action of these DNA repair pathways.

Acknowledgements

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References

1. Ploeg M, Aben KK, Kiemeney LA. The present and future burden of urinary bladder cancer in the world. *World J Urol* 2009;27:289-93.
2. Liu L, Zhang J, Deapen D, *et al.* Cancer in Los Angeles county: incidence and mortality by race/ethnicity 1988-2000. University of Southern California, 2003.
3. Parkin DM, Whelan SL, Ferlay J, *et al.* Cancer. Incidence in Five Continents, Volume VIII IARC Scientific Publications No. 155 Lyon: International Agency for Research on Cancer, 2002.
4. Lerner SP, Schoenberg MP, Sternberg CN. Textbook of bladder cancer. Abingdon, Oxon; Boca Raton: Taylor & Francis, 2006.
5. IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, International Agency for Research on Cancer. Tobacco smoking. Lyon: World Health Organization, International Agency for Research on Cancer, 1986.
6. Freedman ND, Silverman DT, Hollenbeck AR, *et al.* Association between smoking and risk of bladder cancer among men and women. *JAMA* 2011;306:737-45.
7. Scelo G, Brennan P. The epidemiology of bladder and kidney cancer. *Nat Clin Pract Urol* 2007;4:205-17.
8. Gago-Dominguez M, Castelao JE, Yuan JM, *et al.* Use of permanent hair dyes and bladder-cancer risk. *Int J Cancer* 2001;91:575-9.
9. Vineis P, Talaska G, Malaveille C, *et al.* DNA adducts in urothelial cells: relationship with biomarkers of exposure to arylamines and polycyclic aromatic hydrocarbons from tobacco smoke. *Int J Cancer* 1996;65:314-6.
10. Pryor WA, Hales BJ, Premovic PI, *et al.* The radicals in cigarette tar: their nature and suggested physiological implications. *Science* 1983;220:425-7.
11. Maeda H, Sawa T, Yubisui T, *et al.* Free radical generation from heterocyclic amines by cytochrome b5 reductase in the presence of NADH. *Cancer Lett* 1999;143:117-21.
12. Burger MS, Torino JL, Swaminathan S. DNA damage in human transitional cell carcinoma cells after exposure to the proximate metabolite of the bladder carcinogen 4-aminobiphenyl. *Environ Mol Mutagen* 2001;38:1-11.
13. Lin J, Kadlubar FF, Spitz MR, *et al.* A modified host cell reactivation assay to measure DNA repair capacity for removing 4-aminobiphenyl adducts: a pilot study of bladder cancer. *Cancer Epidemiol Biomarkers Prev* 2005;14:1832-6.
14. Stern MC, Lin J, Figueroa JD, *et al.* Polymorphisms in DNA repair genes, smoking, and bladder cancer risk: findings from the international consortium of bladder cancer. *Cancer Res* 2009;69:6857-64.
15. Xing J, Dinney CP, Shete S, *et al.* Comprehensive pathway-based interrogation of genetic variations in the nucleotide excision DNA repair pathway and risk of bladder cancer. *Cancer* 2012;118:205-15.
16. Castelao JE, Yuan JM, Skipper PL, *et al.* Gender- and smoking-related bladder cancer risk. *J Natl Cancer Inst* 2001;93:538-45.
17. Tao L, Xiang YB, Wang R, *et al.* Environmental tobacco smoke in relation to bladder cancer risk-the Shanghai bladder cancer study [corrected]. *Cancer Epidemiol Biomarkers Prev* 2010;19:3087-95.
18. Yuan JM, Wang XL, Xiang YB, *et al.* Preserved foods in relation to risk of nasopharyngeal carcinoma in Shanghai, China. *Int J Cancer* 2000;85:358-63.
19. Edlund CK, Lee WH, Li D, *et al.* Snagger: a user-friendly program for incorporating additional information for tagSNP selection. *BMC Bioinformatics* 2008;9:174.
20. Oliphant A, Barker DL, Stuelpnagel JR, *et al.* BeadArray technology: enabling an accurate, cost-effective approach to high-throughput genotyping. *Biotechniques* 2002;Suppl:56-8, 60-1.
21. Conneely KN, Boehnke M. So many correlated tests, so little time! Rapid adjustment of P values for multiple correlated tests. *Am J Hum Genet* 2007;81:1158-68.
22. Yu K, Li Q, Bergen AW, Pfeiffer RM, *et al.* Pathway analysis by adaptive combination of P-values. *Genet Epidemiol* 2009;33:700-9.
23. Tang W, Fu YP, Figueroa JD, *et al.* Mapping of the UGT1A locus identifies an uncommon coding variant that affects mRNA expression and protects from bladder cancer. *Hum Mol Genet* 2012;21:1918-30.
24. Rothman N, Garcia-Closas M, Chatterjee N, *et al.* A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. *Nat Genet* 2010;42:978-84.
25. Rafnar T, Vermeulen SH, Sulem P, *et al.* European genome-wide association study identifies SLC14A1 as a new urinary bladder cancer susceptibility gene. *Hum Mol Genet* 2011;20:4268-81.

26. Menashe I, Figueroa JD, Garcia-Closas M, *et al.* Large-scale pathway-based analysis of bladder cancer genome-wide association data from five studies of European background. *PloS one* 2012;7: e29396.
27. Kiemeney LA, Thorlacius S, Sulem P, *et al.* Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. *Nat Genet* 2008;40:1307-12.
28. Kiemeney LA, Sulem P, Besenbacher S, *et al.* A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. *Nat Genet* 2010;42: 415-9.
29. Garcia-Closas M, Ye Y, Rothman N, *et al.* A genome-wide association study of bladder cancer identifies a new susceptibility locus within SLC14A1, a urea transporter gene on chromosome 18q12.3. *Hum Mol Genet* 2011;20: 4282-9.
30. Friedberg EC. How nucleotide excision repair protects against cancer. *Nat Rev Cancer* 2001;1: 22-33.
31. Sugasawa K, Ng JM, Masutani C, *et al.* Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol Cell* 1998;2:223-32.
32. Araki M, Masutani C, Takemura M, *et al.* Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. *J Biol Chem* 2001;276:18665-72.
33. Qiao B, Scott GB, Elliott F, *et al.* Functional assays to determine the significance of two common XPC 3'UTR variants found in bladder cancer patients. *BMC Med Genet* 2011; 12:84.
34. Burgers PM. Polymerase dynamics at the eukaryotic DNA replication fork. *J Biol Chem* 2009;284: 4041-5.
35. Wu X, Gu J, Grossman HB, *et al.* Bladder cancer predisposition: a multigenic approach to DNA-repair and cell-cycle-control genes. *Am J Hum Genet* 2006;78:464-79.
36. Huang M, Dinney CP, Lin X, *et al.* High-order interactions among genetic variants in DNA base excision repair pathway genes and smoking in bladder cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* 2007;16:84-91.
37. Figueroa JD, Malats N, Real FX, *et al.* Genetic variation in the base excision repair pathway and bladder cancer risk. *Hum Genet* 2007;121: 233-42.
38. Dogliotti E, Fortini P, Pascucci B, *et al.* The mechanism of switching among multiple BER pathways. *Prog Nucleic Acid Res Mol Biol* 2001; 68:3-27.
39. Wagner U, Bubendorf L, Gasser TC, *et al.* Chromosome 8p deletions are associated with invasive tumor growth in urinary bladder cancer. *Am J Pathol* 1997;151:753-9.
40. Michiels S, Laplanche A, Boulet T, *et al.* Genetic polymorphisms in 85 DNA repair genes and bladder cancer risk. *Carcinogenesis* 2009;30:763-8.
41. Boiteux S, Radicella JP. The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis. *Arch Biochem Biophys* 2000;377:1-8.
42. Lee AJ, Hodges NJ, Chipman JK. Interindividual variability in response to sodium dichromate-induced oxidative DNA damage: role of the Ser326Cys polymorphism in the DNA-repair protein of 8-oxo-7,8-dihydro-2'-deoxyguanosine DNA glycosylase 1. *Cancer Epidemiol Biomarkers Prev* 2005;14:497-505.
43. Yamane A, Kohno T, Ito K, *et al.* Differential ability of polymorphic OGG1 proteins to suppress mutagenesis induced by 8-hydroxyguanine in human cell in vivo. *Carcinogenesis* 2004;25: 1689-94.
44. Hill JW, Evans MK. Dimerization and opposite base-dependent catalytic impairment of polymorphic S326C OGG1 glycosylase. *Nucleic Acids Res* 2006;34:1620-32.
45. Sidorenko VS, Grollman AP, Jaruga P, *et al.* Substrate specificity and excision kinetics of natural polymorphic variants and phosphomimetic mutants of human 8-oxoguanine-DNA glycosylase. *Febs J* 2009;276:5149-62.
46. Kershaw RM, Hodges NJ. Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1. *Mutagenesis* 2012;27:501-10.
47. Wei B, Zhou Y, Xu Z, *et al.* The effect of hOGG1 Ser326Cys polymorphism on cancer risk: evidence from a meta-analysis. *PLoS One* 2011;6: e27545.
48. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125-31.
49. Seo YK, Jeon TI, Chong HK, *et al.* Genome-wide localization of SREBP-2 in hepatic chromatin predicts a role in autophagy. *Cell Metab* 2011;13: 367-75.
50. Chen Y, Hughes-Fulford M. Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator, SREBP2. *Int J Cancer* 2001;91:41-5.

Bohn, Brent 1131

From: Powers, Christina
Sent: Thursday, April 24, 2014 3:44 PM
To: Lee, Janice
Cc: Powers, Christina
Subject: Articles of Potential Interest
Attachments: 2013 Meek WHO IPCS Framework MOA Species Concordance J App Tox.pdf; 2014 Meek MOA Human Relevance Bradford Hill J App Tox.pdf

Hi Janice,

The articles I mentioned in relation to the AOP and susceptible populations manuscript are attached. Much of the discussion is focused on the MOA framework (authors argue that it's synonymous with AOP), but I think several points may be relevant for the paper we've discussed, as well as for the iAs assessment.

The article linked below might also be of interest for the Receptors group more broadly. I recently shared the article with John but am not sure if he's had a chance to pass it on.

Happy to discuss anything further if it's helpful.

Cheers,
Christy

A cross-sectional study of well water arsenic and child IQ in Maine schoolchildren:
http://www.ehjournal.net/content/13/1/23

From: Powers, Christina
Sent: Monday, April 14, 2014 7:48 AM
To: Cowden, John
Cc: Powers, Christina
Subject: Article of potential interest: iAs levels in Maine well water + Child IQ

Hi John,

Hope you had a spectacular weekend and Monday is off to a good start!

I came across the article linked below and thought it might be of particular interest to the DREAM team. I'm sure it will be picked up in the ongoing literature searches, but given the population that the study evaluates we might want to start taking a look sooner than later. Please feel free to share with anyone on the team as you see fit.

Best,
Christy

A cross-sectional study of well water arsenic and child IQ in Maine schoolchildren:
http://www.ehjournal.net/content/13/1/23

Review Article

Journal of Applied Toxicology

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New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis†

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ABSTRACT: The World Health Organization/International Programme on Chemical Safety mode of action/human relevance framework has been updated to reflect the experience acquired in its application and extend its utility to emerging areas in toxicity testing and non-testing methods. The underlying principles have not changed, but the framework's scope has been extended to enable integration of information at different levels of biological organization and reflect evolving experience in a much broader range of potential applications. Mode of action/species concordance analysis can also inform hypothesis-based data generation and research priorities in support of risk assessment. The modified framework is incorporated within a roadmap, with feedback loops encouraging continuous refinement of fit-for-purpose testing strategies and risk assessment. Important in this construct is consideration of dose–response relationships and species concordance analysis in weight of evidence. The modified Bradford Hill considerations have been updated and additionally articulated to reflect increasing experience in application for cases where the toxicological outcome of chemical exposure is known. The modified framework can be used as originally intended, where the toxicological effects of chemical exposure are known, or in hypothesizing effects resulting from chemical exposure, using information on putative key events in established modes of action from appropriate *in vitro* or *in silico* systems and other lines of evidence. This modified mode of action framework and accompanying roadmap and case examples are expected to contribute to improving transparency in explicitly addressing weight of evidence considerations in mode of action/species concordance analysis based on both conventional data sources and evolving methods.

Keywords: key events; mode of action; adverse outcome pathway; human relevance framework; modified Bradford Hill considerations; weight of evidence approach; species concordance analysis; cellular response; tissue response; molecular target

Introduction

The mode of action/human relevance framework was developed in initiatives of the International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) (Boobis *et al.*, 2006, 2008; Sonich-Mullin *et al.*, 2001) and the International Life Sciences Institute Risk Sciences Institute (ILSI-RSI) (Meek *et al.*, 2003; Seed *et al.*, 2005). It derives from earlier work on mode of action in animals by the US Environmental Protection Agency (US EPA, 1996, 2005a) and has involved large numbers of scientists internationally.

Previous development of the mode of action/human relevance framework is described in the publications mentioned above and summarized more recently in Meek and Klaunig (2010). The framework has been illustrated by an increasing number of case studies (more than 30 currently) demonstrating the value of mode of action in evaluating human relevance and life stage susceptibility and guiding dose–response assessment. Documented examples are presented in Table 1. The contribution of the framework has been recognized by the Society of Toxicology, and the framework has been adopted by several international and national organizations and agencies to increase transparency in the assessment of weight of evidence and identification of critical data needs (Meek, 2008, 2009; Meek *et al.*, 2008).

The framework continues to evolve as experience increases in its application to consider systematically the weight of evidence

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Table 1. Case studies illustrating various modes of action and implications for dose–response assessment

Mode of action	Case study	Reference
Tumors of various organs associated with mutagenic modes of action	Ethylene oxide	Meek *et al.* (2003)
	4-Aminobiphenyl	Cohen *et al.* (2006a)
Mammary tumors associated with suppression of luteinizing hormone	Atrazine	Meek *et al.* (2003)
Thyroid tumors associated with increased clearance of thyroxine	Phenobarbital	Meek *et al.* (2003)
	Thiazopyr	Dellarco *et al.* (2006)
Bladder tumors associated with the formation of urinary tract calculi	Melamine	Meek *et al.* (2003)
Liver/kidney tumors associated with sustained cytotoxicity and regenerative proliferation	Chloroform	Meek *et al.* (2003)
Acute renal toxicity associated with precipitation of oxalate	Ethylene glycol	Seed *et al.* (2005)
Androgen receptor antagonism and developmental effects	Vinclozolin	Seed *et al.* (2005)
Nasal tumors associated with DNA reactivity and cytotoxicity	Formaldehyde	McGregor *et al.* (2006)

from traditional and evolving methods for assessing toxicity. This includes explicit consideration of the comparative weight of evidence and associated uncertainties for several options for hypothesized modes of action early and throughout the analysis. The critical relevance of the kinetic and dynamic information considered in the mode of action analysis for subsequent characterization of dose–response relationships for effects considered relevant to humans (Boobis *et al.*, 2009; Julien *et al.*, 2009), including choice of chemical-specific adjustment factors (Boobis *et al.*, 2008), has also been amplified. Experience in mode of action analysis has also been instructive in contextualizing appropriate application of information from evolving methods of toxicity testing at different levels of biological organization as a basis for more efficient testing strategies.

Objectives

This paper has been prepared as an addendum to the previous WHO/IPCS guidance on mode of action/human relevance analysis (Boobis *et al.*, 2006, 2008). While the underlying principles and methodology are similar, the guidance has been updated to reflect recent developments. Some of these developments result from advances in toxicity testing and non-testing methods, and some reflect evolving experience in mode of action/species concordance analysis (additionally referred to herein as mode of action analysis). More detailed information on the nature of systematic hypothesis generation and weight of evidence considerations in mode of action analysis with illustrative case examples is included in the earlier publications referenced in Table 1.

This paper also expands the scope of previous manuscripts to reflect increased understanding of the role of mode of action/species concordance analysis in integrating information from different levels of biological organization. In addition, while early focus of mode of action analysis related to increasing transparency in documenting an operative mode of action with a reasonably high degree of confidence as a basis for risk assessment and regulatory decision-making, the current paper addresses a much broader range of contexts. These include implications for priority setting and testing strategies for both individual chemicals and chemical categories where a less refined analysis and/or higher uncertainty may be acceptable. Summaries of cases selected to illustrate examples of broad application in a research/regulatory context are included here. Readers are referred to the cited documentation for more detailed information on the data analysis for these cases.

Both cancer and non-cancer effects are addressed, in recognition that their separation in earlier publications reflected principally evolving experience in mode of action/human relevance analysis rather than variation in conceptual premise. In fact, mode of action analysis facilitates harmonization of cancer and non-cancer assessment. Harmonization in this context refers to a biologically consistent approach to risk assessment for all endpoints, for which exploration of biological linkages is critical to ensuring maximal utility of relevant information. Often, for example, cytotoxicity in an organ is a critical key event that may lead to an increase in cell proliferation and tumors at the same site.

Background/Terminology

Mode of action, as previously defined, is a biologically plausible series of key events leading to an effect (Sonich-Mullin *et al.*, 2001). Originally, mode of action was considered principally in the context of late-stage key cellular, biochemical and tissue events. A key event is an empirically observable step or its marker, which is a necessary element of the mode of action critical to the outcome (i.e., necessary, but not necessarily sufficient in its own right); key events are measurable and reproducible. The mode of action framework is based, then, on the premise that any human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key events that result in a pathological or other disease outcome. (The term mode of action implies no judgment about adversity of effect, though for risk assessment application, the relevant identified or presumed effects are most often considered adverse.) While originally and often simply conceptualized and illustrated as a linear series of key events, in reality, mode of action involves interdependent networks of events with feedback loops. Disease outcomes are initiated or modified within these networks. Differences in networks between and within human and animal populations account, in part, for interspecies differences and human variability.

Early key events in hypothesized modes of action are most often related to chemical characteristics, i.e., those characteristics of structure and/or physicochemical properties that promote interaction of the substance with biological targets. Later key events are less chemical specific and more often an expected consequence of progression of earlier key events (e.g., regenerative proliferation resulting from cytotoxicity).

An adverse outcome pathway is conceptually similar to a mode of action. It was initially described by the computational ecotoxicology community (Ankley *et al.*, 2010) and has been adopted within an international initiative to document, develop and assess the completeness of potentially predictive tools for adverse ecological and human health effects (OECD, 2012). A focus of adverse outcome pathways is on the initial associated chemically mediated "molecular initiating event," equivalent to an early key event in a mode of action.

The terms mode of action and adverse outcome pathway should be interchangeable, representing essentially the subdivision of the pathway between exposure and effect in either individuals or populations into a series of hypothesized key events at different levels of biological organization (e.g., molecular, subcellular, cellular, tissue) (Fig. 1). (The term toxicity pathway, introduced by the US National Research Council in 2007 [NRC, 2007], essentially focuses on a subset of early events leading to an effect at the molecular and cellular levels. These events can be considered critical upstream elements of a more expansive mode of action description of how a chemical can affect human health.) The distinction between mode of action and adverse outcome pathway is artificial, a result principally of experience in the human health versus ecological communities, though it has sometimes been stated incorrectly that, unlike adverse outcome pathway, mode of action does not extend from the individual to the population level. It should be noted, though, that the term mode of action, *per se*, does not imply adversity of outcome. Mode of action, as defined here, could apply equally well to effects that are not adverse, such as therapeutic interventions or health benefits (e.g., from nutritional supplements). Also, focus on human health risk assessment has traditionally been on (often later) key events that provide quantitative information relevant to intraspecies and interspecies extrapolation and life stage susceptibility for dose–response analysis, compared with the molecular initiating event in ecological health assessment. For this reason, considerations relevant to weight of evidence analysis may differ.

Appropriately, given their conceptual similarity, it has been proposed that the weight of evidence for both hypothesized modes of action and adverse outcome pathways should draw upon modified Bradford Hill considerations (Hill, 1965). This proposal was based on a desire to increase transparency and consistency in organizing, linking and integrating information at different levels of biological organization into a more efficient, hypothesis-driven approach to chemical data generation and assessment and use of non-test (e.g., read-across and grouping of chemicals) and *in vitro* methods.

However, there are a number of limitations that remain to be addressed in the proposed reliance on modified Bradford Hill considerations for documentation of mode of action where focus has been on the molecular initiating event (i.e., structure–activity modeling). For example, weight of evidence for hypothesized modes of action in human health risk assessment has traditionally relied heavily on the modified Bradford Hill considerations of concordance of dose–response relationships between key and end events. In addition, influential in mode of action analysis is specificity, which in this context has related to experimental verification that a key event is causal. And while experience in mode of action analyses for documented (adverse) effects in human health risk assessment can inform consideration of weight of evidence for hypothesized modes of action or adverse outcome pathways, based on early key or molecular initiating events, to date, information on dose–response concordance and specificity has not been available in characterizing weight of evidence for hypothesized adverse outcome pathways. This detracts considerably from transparency in documentation of their supporting evidence.

Mode of Action/Adverse Outcome Pathways—Levels of Biological Organization

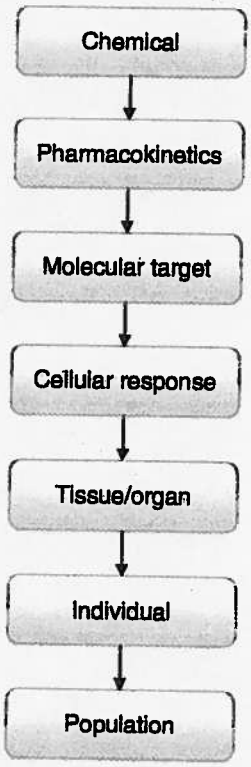

Figure 1. Different levels of biological organization in mode of action analysis. Confidence in an hypothesized mode of action generally increases with increasing evidence at higher levels of biological organization.

Mode of Action Roadmap

There is growing recognition of the need for more efficient methods and strategies to assess the hazards, exposures and risks of the wide array of chemicals to which humans are exposed. This has been reflected in, among others, progressive regulatory mandates in Canada, the European Union and, more recently, the Asian Pacific region to systematically consider priorities for risk management from among all existing chemicals (see, for example, Council of Labor Affairs, Taiwan, 2012; Dellarco *et al.*, 2010; European Commission, 2006; Hughes *et al.*, 2009; Lowell Center for Sustainable Production, 2012; Meek and Armstrong, 2007). This necessitates focus on efficiently prioritized chemicals and endpoints, rather than the traditional time- and resource-intensive series of standard *in vivo* toxicology studies. It also requires the development and integration of information on key events within (hypothesized) modes of action very early in the evaluation process that will enable effective use of data collected from lower levels of biological organization and non-test methods, such as (quantitative) structure–activity relationships ((Q)SAR) and read-across *in vitro* assays.

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Figure 2 presents a "mode of action roadmap" to illustrate the iterative process whereby principles and concepts of mode of action analysis can be applied throughout human health risk assessment, with the extent of the analysis being tailored to the issue under consideration. Critical to this more tailored consideration of appropriate testing and assessment strategies is formal, transparent consultation with risk managers, with public accountability, where possible, for the relevant extent of resource investment to address the problem at hand (i.e., problem formulation).

Problem formulation (Fig. 3), the first step in the roadmap (Fig. 2), involves consideration of the risk management scope and goals in relation to relevant exposure scenarios, available resources, urgency of the assessment and the level of uncertainty that is acceptable. This includes consideration of appropriate methods and endpoints for hazard assessment and a mode of action analysis plan tailored to the nature of the decision to be made. For example, decisions concerning chemical prioritization for testing and/or assessment will likely allow for higher levels of uncertainty than those related to establishing regulatory standards. In problem formulation, then, the complexity of the envisaged mode of action analysis is tailored to the context of decision-making; approaches are necessarily flexible and iterative, permitting efficient identification and generation of the essential information to serve as a basis to assess and manage risks appropriately.

The second step in the roadmap (Fig. 2) is to assimilate and consider, in iterative fashion, information on mode of action in the "Modified framework" (see below). This entails hypothesis-based analysis of the weight of evidence for operative key events based on the modified Bradford Hill considerations and qualitative and quantitative concordance of the key events within and between species (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005). Early consideration of hypothesis-based key events in the mode of action during problem formulation facilitates incorporation of data from different sources and provides a framework by which it can be organized, integrated and linked at different levels of biological organization (Fig. 3). This includes information generated by evolving methods, such as those targeting cell signaling pathways. The

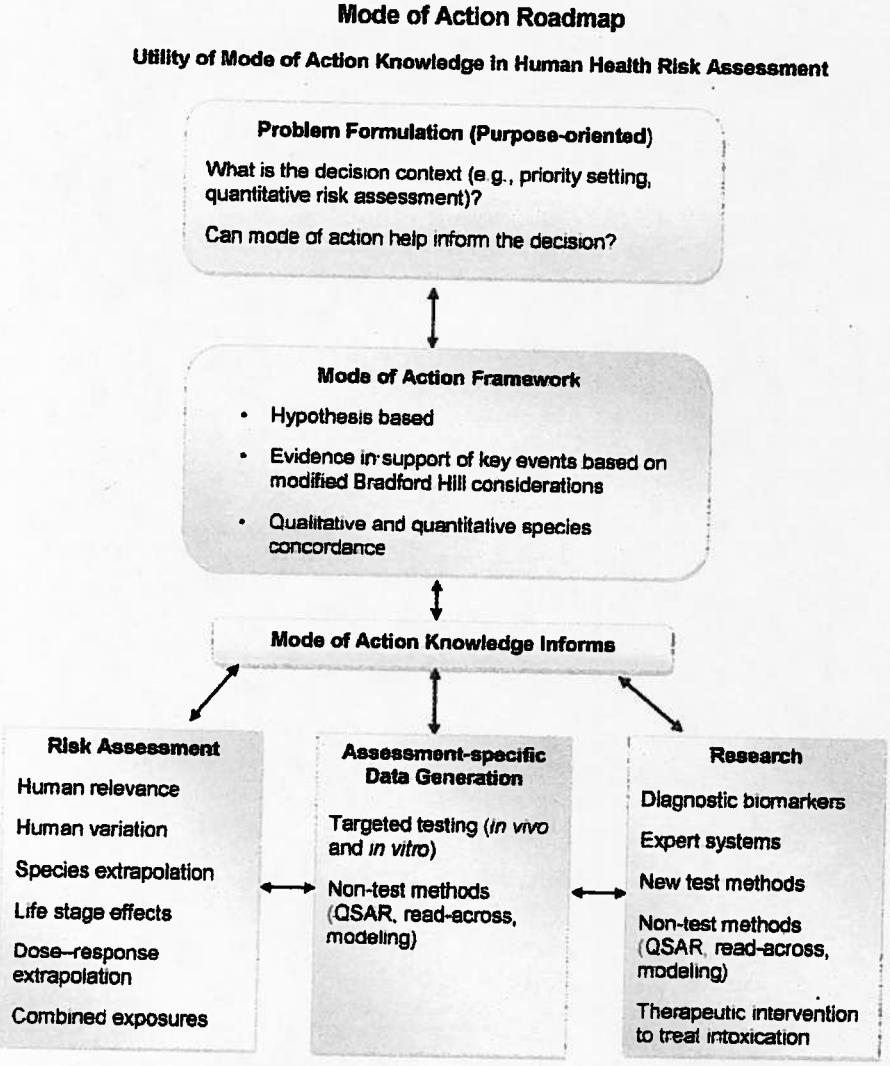

Figure 2. Mode of action roadmap illustrating the use of mode of action knowledge in human health risk assessment. The extent of analysis is tailored to the issue under consideration through iterative analysis and consultation among the assessment, management and research communities.

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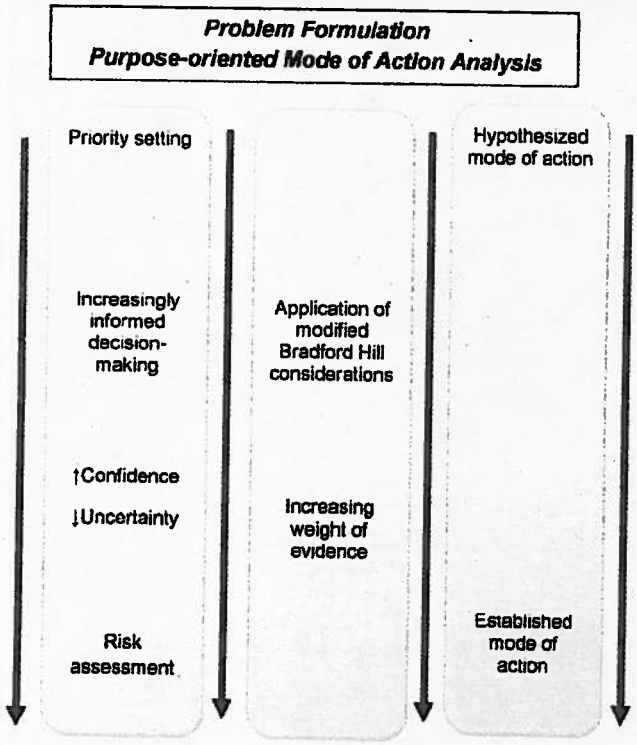


Figure 3. Confidence/uncertainty in "fit for purpose" mode of action/species concordance analysis: correlation of confidence/uncertainty with extent of weight of evidence.

amount of detail and "linearity" characterizing the key events within a hypothesized mode of action can vary as a function of the toxicity of interest, existing knowledge and risk assessment or testing needs.

The mode of action analysis, completed to address the goals outlined during problem formulation, informs one or more of three analytical domains (shown at the bottom of Fig. 2):

(1) risk assessment, including qualitative and quantitative human relevance and variability (e.g., effects at various life stages and within susceptible subgroups), dose–response extrapolation and potential for combined effects of chemicals;
(2) hypothesis-based targeted testing or application of non-test methods to meet the objectives specified in problem formulation, including efficient grouping of chemicals and consideration of read-across, (Q)SAR modeling or appropriate testing within a category approach to fill data needs; and
(3) research priorities relevant to the development of new test and non-test methods, biomarkers and expert systems that feed back to the risk assessment and therapeutic intervention strategies (for intoxication).

As depicted in the roadmap (Fig. 2), mode of action analysis is envisioned as an iterative hypothesis generating and testing process that defines how to assess or test strategically based on risk management needs. As analyses are completed, the problem formulation, testing strategy and risk assessment can be further refined for the decision context.

This iterative process can be illustrated with the following hypothetical example, for which there are considerable data on hazard. While this example draws on a relatively extensive data set, it provides a model for considering significantly fewer data on similar compounds, if they are taken into account from the outset in problem formulation. Initially, a risk manager requests that a risk assessment for the general population be conducted for chemical X, for which exposures of potential concern are those through drinking water. In relatively extensive (traditional) toxicity studies (including a cancer bioassay), chemical X has caused liver tumors in rodents. There is controversy regarding the relevance of this particular tumor type for human health risk assessment, and, based on the preliminary mode of action/species concordance analysis in problem formulation, the risk manager is informed that knowledge of the mode of action of induction of tumors in the relevant dose range could inform conclusions on human relevance. Conduct of appropriate studies to address important data needs and uncertainties in the mode of action analysis can then be considered collectively by the risk manager/risk assessor in a refined problem formulation, depending on resources available and time frame for completion.

If additional generation of data is deemed appropriate, the assessment enters the "research" portion of the roadmap, but with a focused effort on generating data relevant to the mode of action/risk assessment question at hand. The targeted relevant mechanistic data that would inform additional assessment and/or management do not require full knowledge of the mechanism, but rather often quantitative information on determinants of key events, as a basis to predict interspecies differences and human variability better. Upon completion of relevant studies and subsequent mode of action/species concordance analysis, the risk manager is informed of the conclusion (i.e., whether data are considered sufficient to support the hypothesis that the tumors are unlikely to be of relevance to humans).

A potential variant includes the scenario that since the initial problem formulation, the risk manager has become aware that several other related chemicals co-occur with the substance of interest, which may be appropriate for consideration in the same category with chemical X in the risk assessment. The risk manager is informed that the rationale for inclusion of other category members would be strengthened if the same mode of action was suspected; relative potency could then be considered through targeted testing of an early key event. The assessment process now enters the "assessment-specific data generation" portion of the roadmap. Problem formulation can be an iterative process; thus, the results of the targeted testing would further inform the risk manager as to which chemicals within the category are hypothesized to act via the same mode of action, and therefore which should be included for read-across in a combined risk assessment. The assessment process then enters the final "risk assessment" portion of the roadmap.

Modified Framework

The mode of action framework addresses two key questions. The first is whether there are sufficient data to hypothesize, with an acceptable level of confidence, a mode of action for a known or suspected toxicological outcome. The second is the extent to which such a mode of action would, or is likely to, operate in humans at relevant exposure levels (species concordance analysis).

The framework can also be used in two quite different ways, the first reflecting how it was initially developed, for relatively data-rich chemicals. In this case, causal key events related to an observed (adverse) effect associated with a specific chemical exposure are

identified as a basis to utilize available data on kinetics and dynamics maximally to inform relevance to humans and subsequent dose–response analysis; this is referenced below as "Application of the mode of action framework for observed (adverse) effects" and reflects historical experience as is illustrated in many of the case studies currently available. Following problem formulation (Figs 2 and 3), then, a decision may be taken that a mode of action analysis would be of value in addressing an observed toxicological response for which the margin between measures of hazard and estimated human exposure is such that it warrants additional refinement of the assessment.

The second way in which the framework can be applied is based on information on key events from appropriate *in vitro* and *in silico* systems to predict and assess potential modes of action and potential consequent (adverse) effects (referenced below as "Application of the mode of action framework in hypothesizing (adverse) effects"). The outcome of such an analysis may be the development of a plausible case to predict an (adverse) effect based on knowledge of putative key events or, alternatively, the probable exclusion of certain (adverse) effects, based on an absence of a likelihood of perturbation leading to relevant key events.

In this context, mode of action comprises a series of causally associated key events leading to, potentially leading to or hypothesized to lead to an (adverse) effect. Hence, there can be only one mode of action for one chemical or group of chemicals leading to a specified effect under a given set of conditions. However, different chemicals, or the same chemical under different conditions (e.g., at higher doses or concentrations), may produce the same effect via different modes of action. An example would be the generation of site of contact tumors in the nasal cavity. One chemical may produce such an effect through cytotoxicity and subsequent cell replication promoting spontaneous mutations, another through DNA reactivity leading to gene mutations promoted by regenerative proliferation secondary to cytotoxicity, and a third through interaction with DNA leading to early mutations. In addition, early key events in competing pathways may, or often, converge to produce the same late key event (and outcome). Each mode of action comprising a series of key events for a given response will be different, but some of the key events may be common to other modes of action leading to the same response. The nature of the key events involved will have an impact on the shape of the dose–response curve and on interspecies and intraspecies differences.

The modified mode of action framework is outlined in Fig. 4 and explained in further detail below.

Application of the Mode of Action Framework for Observed (Adverse) Effects

Only this first approach was addressed in the previous descriptions of the WHO/IPCS/ILSI-RSI mode of action/human relevance framework (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005), from which further detailed information can be obtained. Extension of the approach through application to help construct more predictive groupings of chemicals was subsequently highlighted in Carmichael *et al.* (2011). A key aspect of the approach, as illustrated through case studies, is that there should be an unequivocal effect to address before embarking on a mode of action analysis. Hence, problem formulation will

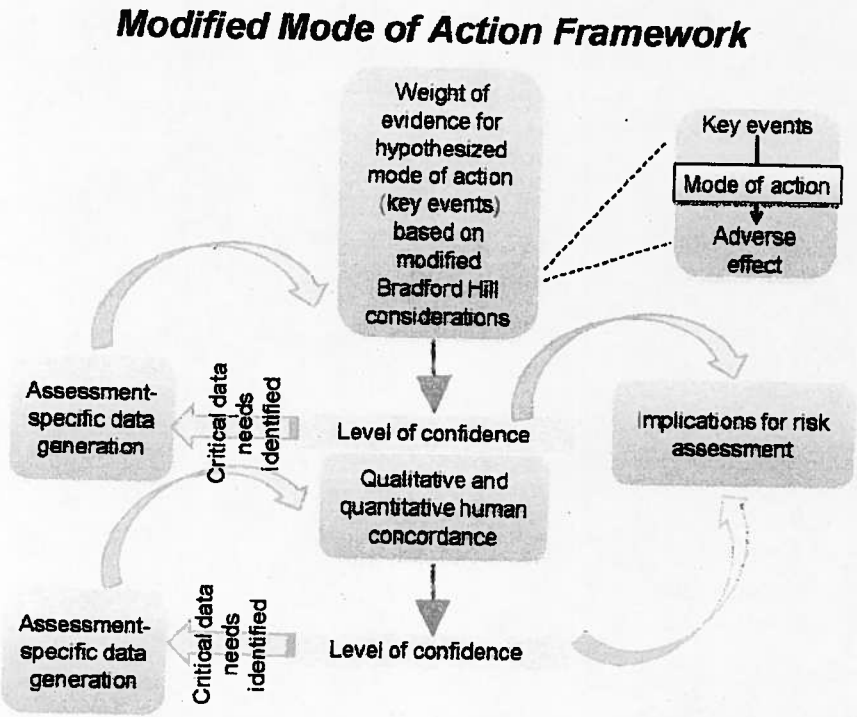


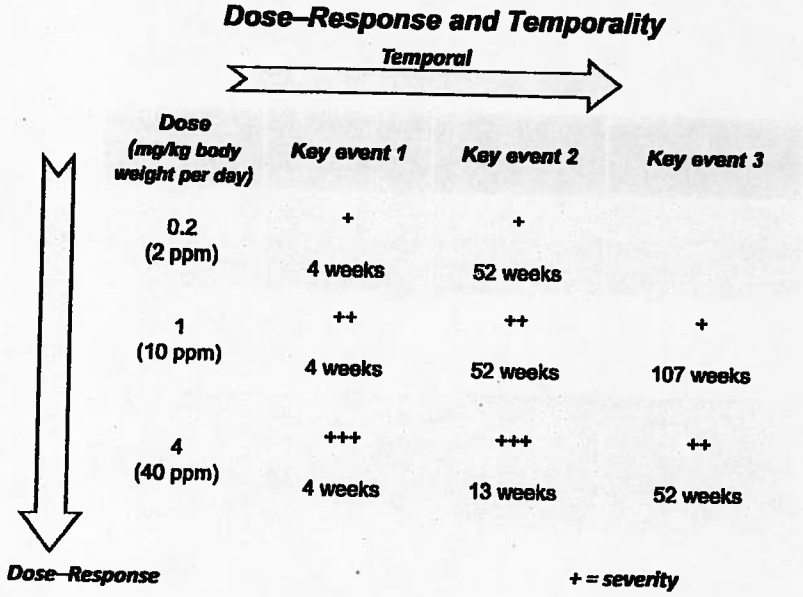
Figure 4. Modified mode of action/human relevance framework and its relation to data needs identified and risk assessment. The application of the framework to assess for observed (adverse) effects and in hypothesizing (adverse) effects is illustrated. The iterative nature of the analysis and the importance of expressing uncertainty are also highlighted.

have identified the (critical) effect(s) of concern to be considered in the analysis.

In general, mode of action analysis applies to a single effect in a single tissue. In essence, there is one mode of action leading to an effect of interest in the relevant organ for a given substance. This mode of action entails several key events, each of which may result from different (sometimes) competing mechanisms and/or pathways, although these converge at a late stage to produce the (adverse) effect. It is important, then, to robustly synthesize available information based on multidisciplinary input in hypothesizing potential modes of action. In addition, in the absence of information to the contrary, site concordance between animals and humans is generally assumed, at least as an initial premise. This is often the case, for example, for many non-genotoxic carcinogens that act through perturbation of physiological processes. Similarly, for many non-cancer

Modified Bradford Hill Considerations

- Concordance of dose–response relationships between key and end events
  - Dose–response relationships for key events would be compared with one another and with those for endpoints of concern
    - Are the key events always observed at doses below or similar to those associated with the toxic outcome?
- Temporal association (time)
  - Key events and adverse outcomes would be evaluated to determine if they occur in expected order



- Consistency and specificity
  - Is the incidence of the toxic effect consistent with that for the key events?
    - i.e., Less than that for the key events?
  - Is the sequence of events reversible if dosing is stopped or a key event prevented?
- Biological plausibility
  - Is the pattern of effects across species/strains/systems consistent with the hypothesized mode of action?
  - Does the hypothesized mode of action make sense based on broader knowledge (e.g., biology, established mode of action)?

Figure 5. An illustration of the modified Bradford Hill considerations for weight of evidence of hypothesized modes of action. The illustration represents evolution of these considerations based on increasing experience in application in case studies and training initiatives internationally. Specific questions being addressed by each of the considerations are offered as a basis potentially to increase common understanding and consistency in their application in mode of action analysis.

Comparative Weight of Evidence for Hypothesized Modes of Action: Cytotoxic Mode of Action Example Summaries

Modified Bradford Hill consideration	Supporting evidence	Potentially inconsistent evidence
Dose–response Temporal concordance	Metabolism, cytotoxicity, proliferation precede tumors; tumors observed only at cytotoxic doses (benchmark dose analysis) *(quality based on nature and number of studies)*	Tumors observed at doses lower than those at which key events observed
Consistency, specificity	Consistency in repeated studies and different labs and across species, sexes, routes and levels of biological organization *(#s)* correlating with extent of metabolism. No adverse effects without relevant enzyme in null mice. Incidence of tumors less than that for key events and tissue recovery in reversibility studies.	Incidence of tumors greater than that for key events
Biological plausibility	Consistency with state of knowledge on cancer	

Comparative Weight of Evidence for Hypothesized Modes of Action: Mutagenic Mode of Action[a] Example Summaries

Modified Bradford Hill consideration	Supporting evidence	Potentially inconsistent evidence
Dose–response Temporal concordance	Dose–response and temporal pattern for genotoxicity and tumors consistent with the compound acting via a mutagenic mode of action	Parent compound negative for mutation in a range of *in vitro* and *in vivo* bioassays *(quality based on nature and number of studies)*
Consistency, specificity	Evidence in a range of well conducted bioassays that mutation is an important early key event (e.g., occurs early and at relevant doses)	The pattern of genotoxicity results inconsistent with what would be expected for the hypothesized mode of action (e.g., not mutagenic in a range of assays; metabolite induces mutation at cytotoxic doses)
Biological plausibility	Pattern of results for genotoxicity consistent with that observed for chemicals known to act via a mutagenic mode of action	Pattern of results for genotoxicity inconsistent with that observed for chemicals known to act via a mutagenic mode of action

[a]Where mutation is an early and influential key event.

Figure 6. An example of comparative weight of evidence for hypothesized cytotoxic and mutagenic modes of action. Information in each of the columns provides an overview of the extent and nature of the available data and its cohesiveness. Particularly important in interpretation of relative weight of evidence is the nature and extent of data that may be inconsistent with a hypothesized mode of action. In this particular case, the extent of inconsistent data is considerably less for a hypothesized mode of action where mutation is likely to be secondary to cytotoxicity than for a mutagenic mode of action (i.e., where mutation is an early and influential key event). Indeed, the pattern of data on genotoxicity is completely consistent with a cytotoxic mode of action. This would lead to the conclusion that there is greater confidence in the chemical acting by a cytotoxic than by a mutagenic mode of action.

Concordance Table with Dose–Response

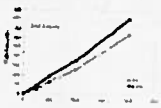
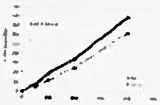
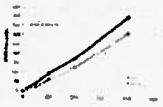
Key event / adverse outcome	Qualitative species concordance	Evidence base	Quantitative species concordance	Quantitative dose–response
Metabolism by cytochrome P450 2E1	Relevant enzyme in kidney and liver of humans	Considerable in animals; limited but relevant to humans	Physiologically based pharmacokinetic model incorporating metabolic rates, enzyme affinities and distribution based on *in vitro* human data supported by *in vivo* data	
Sustained cell damage and repair (cytotoxicity, proliferation)	Liver and kidney target organs in humans	Considerable in animals; possible in humans, but limited data	No data	
Liver and kidney tumors	Possible in humans	Considerable in animals; highly plausible in humans	No data	

Figure 7. An illustration of a concordance table including dose–response curve. The kinetic and dynamic data considered in assessment of mode of action are directly relevant to dose–response analysis, which takes into consideration dose–response relationships for each of the key events.

endpoints, site concordance between test species and humans is a reasonable first assumption, based on considerations of biological plausibility and chemical-specific mechanistic data.

However, there are exceptions to this general principle. Consistent with species- and tissue-specific variation in metabolic activation and detoxification, site concordance for DNA-reactive carcinogens or other effects for which metabolism is critical is often poor. Similarly, for some non-cancer effects induced through a pleiotropic response, such as those that are endocrine mediated, site concordance should not be assumed, but rather considered, based on available mechanistic data and knowledge related to biological plausibility.

These possibilities would need to be scoped at the outset of any mode of action analysis. In such cases, it may be that mode of action analysis would benefit from considering multiple sites in the same evaluation. However, care must be taken to ensure that the mode of action for each effect is likely to be the same, which will not always be the case.

Mode of action analysis relies upon biological plausibility and coherence. The weight of evidence for a hypothesized mode of action is addressed based on the Bradford Hill considerations, proposed originally to examine causality of associations observed in epidemiological studies, but later modified in WHO/IPCS and ILSI-RSI publications on the mode of action/human relevance framework (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005) and additionally evolved, here. The original templates for consideration of the weight of evidence for a hypothesized mode of action were based on consideration of traditional measures of toxicity, such as biochemical and histopathological parameters in experimental animals. These templates have been adapted here (Figs 5–7) to reflect additional experience gained in the application of the framework in an appreciable number of case studies over the past decade and as a basis potentially to encompass additional early key events from evolving methods to reliably predict human health outcomes. Based on this experience, robust consideration of dose–response relationships and temporal concordance for early key events will be important in documenting weight of evidence for proposed adverse outcome pathways.

Relevant considerations include dose–response relationships and temporal concordance between specified key events and outcome, consistency (of, for example, the incidence of key events and outcome and changes in causally associated key events), specificity (in the context of essentiality of key events and reversibility) and biological plausibility, based on coherence with the state of knowledge.

In relation to dose–response relationships and temporal concordance, a key event cannot play a role in an (adverse) effect if it is manifest only after toxicity has occurred or if it occurs only at doses higher than those inducing toxicity. The same applies to late key events relative to early key events. There is often a close relationship between dose and time dependency, so that the higher the dose, the earlier a key event is observably affected, and vice versa. This pattern of dose–response and time–response relationships can be invaluable in assessing weight of evidence for a hypothesized mode of action and its key events or how different key events are interrelated. Systematic consideration of dose–response relationships and temporal concordance between key events and (adverse) effects, as illustrated in Fig. 5, encourages early assimilation of relevant information from the broader database of both short- and long-term studies, or from different non-animal test systems, in a mode of action context.

More detailed discussion on all of the modified Bradford Hill considerations when applied in the mode of action analysis for observed (adverse) effects is provided in previous publications on the mode of action/human relevance framework and will not be repeated here. Application and weighting of these considerations continue to evolve as a basis to additionally increase consistency and transparency in assessing weight of evidence in mode of action/species concordance analysis.

It is essential at the outset of mode of action/species concordance analysis that all reasonably plausible modes of action be considered. These include those modes of action that have previously been associated with the relevant effect and any series of key events that logically presents because of available experimental information. The case for each plausible mode of action should be evaluated systematically from the outset, using modified Bradford Hill considerations.

Weight of evidence for alternative hypotheses should be considered and assessed comparatively. Figure 6 illustrates such an evaluation. Based on relative weight of evidence, it can be determined whether one mode of action could be considered with reasonable certainty to explain the (adverse) effect. Where it is not possible to exclude one or more modes of action, critical data needs could be identified as a basis to inform relevant research that could reduce uncertainty concerning the causal key events within a mode of action, depending on the needs and urgency of the assessment as considered in problem formulation.

The degree of confidence in the outcome should be specified, and each step in the mode of action analysis should be accompanied by a list of the critical uncertainties (i.e., lack of knowledge) and associated data needs, prioritized on the basis of their likely impact, if filled, on weight of evidence and implications for subsequent dose–response analysis.

The comparative analysis of weight of evidence for hypothesized modes of action based on the modified Bradford Hill considerations is followed by statements on the likelihood of each being operative to induce the critical effect. Alternatively, depending on the needs and urgency of the assessment addressed in problem formulation, plausible modes of action should be considered as a basis to contrast strengths and weaknesses of different approaches to quantification of interspecies and intraspecies extrapolation in dose–response modeling. This enables risk managers to distinguish best-supported options (i.e., those that are most certain), which is critical in increasing transparency in separating science judgment (i.e., considerations based on experienced consideration of the relevant science base) from science policy determinations (e.g., embedded conservatism in human health risk assessment, incorporated to increase public health protection). Characterization of this nature also contributes to consistency across weight of evidence considerations in different mode of action analyses.

An important objective of framework analysis, then, is the description of the critical sources of uncertainty and characterization of their impact on conclusions concerning weight of evidence for various hypothesized modes of action and their relevance to humans, as a basis particularly for identification of priorities for generation of more or better data. Sensitivity of the estimate to various assumptions can also be tested, and/or available quantitative data relevant to key uncertainties can be analyzed.

Following mode of action analysis and consideration of the associated uncertainties, several outcomes are possible, as illustrated in Fig. 4. There may be sufficient information to conclude that a hypothesized mode of action is supported by available evidence to explain the effect of concern and that key events for this mode of action have been clearly identified. Where there is insufficient information to reach a conclusion with adequate confidence that a hypothesized mode of action explains the (adverse) effect of concern, appropriate research to address identified critical data needs should provide suitable information to enable confirmation or otherwise of the hypothesized mode of action, through iterative application of the framework. Finally, it may be that at the conclusion of the analysis a hypothesized mode of action is rejected and no other mode of action logically presents itself. In such instances, it may

be necessary to proceed with the risk assessment empirically, using relevant information that has been obtained during the analysis of the mode of action – for example, dose–response and time–response information on the endpoint itself, or relevant kinetic and dynamic data.

An important objective of mode of action analysis is to identify those key events that are likely to be most influential in determining potential qualitative and quantitative differences within and between species – that is, key events that are dose and rate limiting. This is addressed in species concordance analysis and is illustrated in Fig. 7. Where it has been possible to conclude that a hypothesized mode of action is adequately supported by the available information with an acceptable level of confidence, it is necessary to consider the extent to which such a mode of action would, or is likely to, operate in humans. Species concordance analysis starts with a statement on the level of confidence in the weight of evidence for the hypothesized mode of action under consideration and associated uncertainties. The extent of this analysis is necessarily dependent upon the test system(s) in which key events have been measured, being less for those that best represent humans.

Consideration of mode of action also enables identification of early events or indicators of susceptibility that could be measured in humans (i.e., biomarkers); for example, if there is sufficient information to support early key events such as metabolic activation to a reactive metabolite, this directs attention to the relevant parameters in humans, as a basis to predict interspecies (based on comparison of the relevant parameters between humans and animals, scaled as appropriate) and intraspecies differences (based on consideration of the relevant parameters within different subgroups of the population). Consideration of potential key events also contributes to identification of any specific subpopulations (e.g., those with genetic predisposition or life stage differences) that may be at increased risk.

Assessment of concordance is accomplished by systematic consideration of the nature of the key events between and within species, taking into account both chemical-specific and more generic information, such as anatomical, physiological and biochemical variations. Concordance is considered both qualitatively and quantitatively (Fig. 7). On rare occasions, it may be possible to conclude that a mode of action identified in studies in animals is not relevant to humans because of profound qualitative differences identified in experimental investigation; for example, the molecular target necessary for a key event is not present in humans, and there is no functional equivalent. An example would be α_{2u}-globulin, which plays a key role in the renal carcinogenicity of D-limonene (see Case example 1) (Meek *et al.*, 2003). Alternatively, and very infrequently, quantitative differences in key events may be so great as to render the mode of action not relevant to humans at any conceivable exposure to the substance.

Case example 1: Lack of human concordance

D-Limonene provides an example of a data-rich case example for which the mode of action has been established with confidence in the animal model and extensive data are available to demonstrate that it is not relevant to humans (Meek *et al.*, 2003).

Hypothesized key events in the mode of action for species- and sex-specific kidney tumors in male rats were the formation of a stable intermediate, D-limonene-1,2-epoxide, which binds to a protein, α_{2u}-globulin, which accumulates in the renal proximal tubule cells, leading to nephropathy and cellular proliferation, and subsequently tumors, at this site following chronic exposure. There is strong evidence that female rats, laboratory mice and other strains of rats for which there is no evidence of D-limonene-related renal toxicity or tumors do not synthesize or express α_{2u}-globulin.

Consideration of the relevance to humans of the key events leading to renal tumors in the male rat model identified the expression of either α_{2u}-globulin or a homologous protein in humans as critical. After an exhaustive analysis, no protein capable of binding to D-limonene-1,2-epoxide could be identified from human kidney, and therefore it could be concluded that the mode of action leading to kidney tumors in the male rat was not likely to be operable in humans.

This is a rare example of a distinct qualitative difference between the animal model and humans, allowing the possibility to conclude that a mode of action is not relevant to humans. However, it is quite unusual to be able to demonstrate such a qualitative difference. Rather, in the vast majority of cases, such differences will be quantitative, and likely differences in sensitivity of response between animals and humans identified in the mode of action analysis would be taken into account in the subsequent dose–response analysis.

If the weight of evidence for the hypothesized mode of action is sufficient and its relevance for risk assessment cannot be excluded, the implications for dose–response analysis and population variability are considered in the context of identified kinetic and dynamic data. Figure 7 indicates the relevance of delineation of key events in hypothesized modes of action considered to operate in humans in subsequent dose–response analysis. In fact, there is a dose–response curve for each of the key events, and risk for the human population is best predicted on the basis of those key events (or a combination thereof) that are likely to be most influential in impacting or preventing risk, taking into account potential interspecies and interindividual differences in kinetics and dynamics as considered in the species concordance analysis. Reliance on earlier key events offers the potential to better characterize and/or acquire data on effects at lower doses or concentrations in human tissues or populations, which are more relevant for risk assessment. It also contributes to the development of more relevant and informative data for human life stages and subpopulations. For Case example 2, these data could be used additionally in quantitative species concordance analysis, with implications for subsequent dose–response analysis, the identification of critical data needs and the contribution of evolving methods – in this case, well-designed genomic studies – see "Application of the mode of action framework in hypothesizing (adverse) effects" below (see also Table 2).

Case example 2: Use of kinetic and dynamic data in species concordance analysis and implications for dose–response analysis – Contribution of well-designed genomic studies

This example illustrates the manner in which kinetic and dynamic data may potentially inform quantitative concordance analysis, including interspecies variation and human

variability and, subsequently, dose–response analysis and extrapolation. The example also illustrates how mode of action/species concordance analysis informs meaningful generation of critical data relevant to risk assessment, including that from evolving methods.

Cacodylic acid (dimethylarsinic acid) is a pesticide that causes dose-related increases in the incidence of bladder tumors in rats, but not mice (Cohen *et al.*, 2006b, 2007; US EPA, 2005b). Incidence is increased significantly only at the highest administered dose levels. The parent compound undergoes reductive metabolism to a toxic metabolite, and observed damage to urinary epithelial cells correlates with this pathway (see Cohen *et al.*, 2006b; US EPA, 2005b). The levels of toxic metabolite are significantly increased at doses causing cytotoxicity, proliferative regeneration and bladder tumors. The weight of evidence from critically evaluated data from a wide range of assays both *in vitro* and *in vivo* indicates that the parent compound is not mutagenic, but that the active metabolite is clastogenic at high concentrations or doses. The concentration–response relationships for cytotoxicity associated with the active metabolite were similar in *in vitro* studies in bladder cells of rats and humans. Because of toxicokinetic differences, the toxic metabolite is expected to form at a lesser amount in human urine compared with rats (Cohen *et al.*, 2006b; US EPA, 2005b).

Application of the modified Bradford Hill considerations supported the weight of evidence for the hypothesized key events in the mode of action, which included reductive metabolism and cytotoxicity and proliferative regeneration leading to bladder tumors (Cohen *et al.*, 2006b; US EPA, 2005b). Weight of evidence considerations included a thorough analysis of dose–response relationships and temporal concordance as determined from benchmark dose analyses of a range of *in vivo* studies of different durations. This does not imply a 1: 1 correlation of the incidence of early and late key events (rather, the incidence of early key events is expected to be higher), as key events are essential, but not necessarily sufficient in their own right.

Qualitative and quantitative concordance analysis based on relevant kinetic and dynamic data indicated that these effects are relevant to humans and that quantitative differences would most likely be related to extent of delivery to the target organ of the toxic metabolite and variations in sensitivity of the bladder to damage induced by this metabolite. Chemical-specific adjustment factors could then be derived from a physiologically based pharmacokinetic model incorporating metabolic rates, enzyme affinities and distribution based on *in vitro* human data supported by *in vivo* data and quantitative reflection of the similarity in sensitivity to the active metabolite between the rat and human bladder in *in vitro* studies.

The mode of induction of bladder tumors was deduced principally based on key cytological and biochemical events in mechanistic studies from experiments designed to address critical aspects of both the mode of action and species concordance analysis. The results of genomic studies indicated that similar networks were altered in rat and human urothelial cells exposed to the active metabolite at doses similar to those in urine at which tumors were observed in the critical bioassays. The concordance table in Table 2 outlines confidence/uncertainties in the mode of action/species concordance analysis.

Mode of action analysis also contributes to the interpretation of relatively extensive epidemiological data sets. For example, information on key events in mechanistic studies can contribute to better understanding of expected (not necessarily similar) target organs in humans. This is relevant to the interpretation of negative epidemiological data based on their power to detect the most likely site of damage in humans taking into account mode of action and interspecies differences in key determinants of key events. It also contributes to the selection of appropriate biomarkers of effect in epidemiological studies and to understanding of variations between life stages and subgroups of the human population (see Case example 3).

Case example 3: Role of mode of action analysis in the evaluation of epidemiological data

This case example illustrates the contribution of mode of action analysis when there is substantial human evidence.

Associations between ambient particulate matter exposures and increased cardiovascular mortality were first observed in epidemiological studies without support from animal bioassays, which led to skepticism concerning causality due to the lack of mechanistic underpinning. Subsequent mode of action studies shed light on key events in cardiovascular injury in humans exposed to particulate matter and elucidated interspecies differences and human variability in dosimetry and sensitivity (US EPA, 2009b).

Particulate matter induces adverse effects on the cardiovascular and cerebrovascular systems, such as thrombosis, plaque rupture, myocardial infarction and stroke, via reactive oxygen species, which appear to trigger systemic inflammation through the action of cytokines and other soluble mediators. In general, systemic inflammation is associated with changes in circulating white blood cells, the acute phase response, procoagulation effects, endothelial dysfunction and the development of atherosclerosis. The time course of these responses varies according to the acute or chronic nature of the particulate matter exposure; chronic exposures may also lead to adaptive responses.

If there is appreciable uncertainty about the relevance or applicability of a mode of action, but critical data needs can be identified, it may be possible to obtain such information through conduct of appropriate studies. Table 2 includes the concordance analysis for the example included in Case example 2, illustrating principal areas of uncertainty, where generation of additional data might meaningfully inform the risk assessment.

If it is not possible to establish whether a mode of action would, or is likely to, operate in humans with an acceptable level of confidence, but there is a pressing need for risk management decisions because of the urgency or the nature of the problem, knowledge of dose–response relationships and variability across species may still be of value in later stages of the risk assessment.

The conclusions of the concordance analysis should be accompanied by consideration of associated uncertainty and a statement on the level of confidence that a mode of action would, or is likely to, operate in humans.

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Table 2. Concordance analysis of key events in the mode of action associated with induction of bladder tumors in rats by cacodylic acid (Cohen et al., 2006b; US EPA, 2005b)

Key event	Qualitative concordance		Quantitative concordance	Confidence/uncertainty
	Rats	Humans		
Reduction of cacodylic acid (dimethylarsinic acid, or DMA^V) to the highly cytotoxic metabolite, dimethylarsinous acid (DMA^{III}), in urine	Yes: *In vivo* studies detecting DMA^{III} in urine at concentrations that would produce cytotoxicity after DMA^V is administered.	Plausible: Evidence following DMA^V exposure too limited to draw conclusions, but DMA^{III} shown to be present following human exposure to inorganic arsenic.	Formation of less DMA^{III} in urine of humans compared with rats. Significant levels of additional metabolite trimethylarsine oxide (TMAO) in rodents; detected in humans only at very high doses of inorganic arsenic. DMA^V is a poor substrate for the arsenic(III) methyltransferase (AS3MT) in humans. Variation between humans and rats in transport of DMA^V across cell membranes. Similar magnitude of response of human and rat epithelial cells to DMA^{III}. Interspecies differences could be taken into account in dose–response analysis through physiologically based pharmacokinetic modeling and use of chemical-specific adjustment factor for dynamics.	Considerable evidence in animals; limited in humans.
Urothelial cytotoxicity	Yes: Scanning electron micrographs of rat urothelium; *in vivo* cytotoxicity findings correlate closely with *in vitro* studies.	Human evidence from *in vitro* studies of urothelial cells, potential to occur *in vivo* in humans if sufficient DMA^{III} is formed.		Considerable consistent evidence that the metabolite leading to urothelial cytotoxicity is DMA^{III} and that cytotoxicity is a rate-limiting key event; quantitative species differences in key events (mode of action) can be taken into account.[a]
Regenerative urothelial proliferation	Yes: *In vivo* 5-bromo-2'-deoxyuridine labeling index data.	No human evidence, but potential to occur in humans if sufficient cell killing is produced and sustained.		Considerable evidence in animals, although some inconsistencies in the data that can be accounted for by variability across different laboratory studies.
Development of urothelial tumors	Yes: Responses in rats but not mice.	No epidemiological data: Only if humans were exposed to doses of DMA^V that are sufficiently high to lead to cytotoxic levels of DMA^{III} in the urine.		Strong and consistent evidence supporting the sequence of key events postulated for the development of rat bladder tumors. Good understanding of species differences impacting key events. Evidence in humans is weak. Mode of action is qualitatively plausible in humans, presuming sufficient DMA^{III} is present in the urine.

[a]Though the biochemical target for cytotoxicity is not understood, this information is not essential for the mode of action.

Application of the Mode of Action Framework in Hypothesizing (Adverse) Effects

Lessons learned in mode of action/species concordance analysis for identified effects are also relevant to its application where the (adverse) effect is not demonstrated but could potentially be presumed based on measurement of putative early key events in established modes of action, taking into account lines of available evidence.

Thus, hypotheses about the key events that can lead to the observed (adverse) effect of concern are developed. In contrast, one can also develop hypotheses of potential (adverse) effects that may be triggered by observed putative early key events, based on previous generic knowledge on documented modes of action. Both approaches involve an iterative process of hypothesis testing and data generation.

In this approach, the objective is to identify those modes of action that could plausibly arise from the (series of) key events identified, either because of previous knowledge of their involvement in a mode of action (e.g., for related chemicals for which there are more data) or because a plausible case can be made on the basis of existing biological understanding that such (a series of) events or perturbations may reasonably lead to (adverse) outcomes under certain time- and dose-dependent conditions. The methods used for evaluating putative modes of action will be fit for purpose, which will not necessarily involve one-for-one validation against existing *in vivo* methods. Thus, at the outset, consideration of potential key events in the mode of action plays an integral role both in the choice of experimental methods (*in vivo*, *in vitro* or *ex vivo*) and in data interpretation. Based on the understanding of the causal linkage of putative key events (either observed or anticipated), hypotheses of the likely potential effects of exposure to a chemical are developed in mode of action analysis. Thus, the modified Bradford Hill considerations are just as applicable here, but are not yet well tested.

In terms of quantitative dose–response assessment of the key events, a critical factor is extrapolation of the effect levels *in vitro* or predicted *in silico* to target tissue concentration *in vivo* – for example, by using physiologically based toxicokinetic modeling (referenced as quantitative *in vitro* to *in vivo* extrapolation modeling). Thus, a key consideration is target tissue concentration of the toxicologically active moiety. This approach lends itself well to identification of the causative agent (i.e., parent or metabolite) and readily enables qualitative and quantitative information to be obtained on the enzyme reactions involved. It may be possible to discount human relevance of some putative modes of action based on the margin between effect levels *in vitro* and anticipated target tissue concentrations *in vivo*. This may be particularly important in the short term, when there is substantial uncertainty about the significance of weak signals obtained using *in vitro* methods.

As discussed above, confidence in a mode of action postulated on the basis of putative early key events identified using non-animal methods will depend on the weight of evidence linking these key events with a mode of action for an adverse response from previous studies and on the ability to "calibrate" quantitative changes in the key event against a degree of change known to have adverse consequences. An example would be inhibition of an enzyme involved in neurotransmitter synthesis or degradation. The extent to which this enzyme needs to be inhibited to produce adverse consequences may be known from studies *in vivo* and could then be used to calibrate such changes determined *in vitro* or predicted *in silico*. Integral to this would be knowledge of the extent to which adaptive mechanisms operating *in vivo* are functional *in vitro* or included in the *in silico* model systems.

Formal analysis of site concordance for key events may not be necessary in this approach. Similar to the mode of action analysis for observed (adverse) effects, data may have been generated in tissue-specific model systems or may reflect site-specific key events. Prediction of likely site of effect will require additional considerations, such as the uptake and disposition of the chemical and the activity of causal pathways in different tissues and cell types. For example, if toxicity depends in part upon transport into the target cell to reach a critical concentration, the presence of the transporter in different cell types would be a key consideration in assessing potential site specificity. Similarly, if one of the key events involved inhibition of a specific potassium channel, the tissue distribution of this ion channel would be an important factor in assessing site specificity. Eventually, as knowledge of the biology of the causal pathways increases, it may be possible to use a systems approach to predict likely affected tissues.

Critical to interpretation of data obtained using non-animal methods will be the model system in which information on putative early key events was obtained and whether coverage of more than one key event would be expected. Some key events may be assessed individually (e.g., using *in silico* approaches to predict binding affinity to a receptor), whereas others may be assessed in a more integrated system (e.g., cytotoxicity in a metabolically competent cell system). Alternatively, high-content analysis and bioinformatics may be used to identify those pathways affected by a substance.

In the case of a well-established mode of action, the focus is on determining whether the measured key events provide sufficient evidence to accept the plausibility for the (adverse) outcome without necessarily generating *in vivo* data specifically to demonstrate the (adverse) outcome. Where the mode of action has not previously been established, the possibility that a plausible case can be made because of existing biological understanding should be addressed. Failing this, the likely outcome of such an analysis is the generation of a hypothesis for a possible (adverse) effect, which can then be tested *in vivo*. In any event, once a mode of action is established, the key events are known a priori and can then be assessed *in vitro* or *in silico*. Thus, by understanding the likelihood of effects (i.e., initiation of a toxicity pathway) at lower levels of biological organization (e.g., from SARs and *in vitro* models), it can be determined if more expensive and time-consuming testing at higher levels of biological organization (i.e., *in vivo*) is needed, contributing to increasing efficiency in hazard testing of chemicals. Viewed from the opposite perspective, certain *in vivo* testing could be eliminated for substances that show no potential to initiate the chain of events comprising the mode of action for an (adverse) outcome at environmentally relevant concentrations. In other words, tailored testing can be developed according to screening outcomes indicating the potential for (adverse) effects (see Case example 4).

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Case example 4: Use of mode of action analysis to guide development of more efficient testing strategies

Concepts of mode of action analysis are also helpful in guiding developments in the replacement of *in vivo* toxicity testing.

Modes of action can be hypothesized based on reference chemicals/pharmaceuticals where the sequence of key events leading to a specific (adverse) effect is known at a sufficient level of detail, as a basis to facilitate identification of the characteristics and requirements of *in vitro* systems and *in silico* models that could predict early and subsequent rate-limiting key events in an integrated manner. Once dose–response relationships between the key events measured *in vitro* and biomarkers of response and ultimately adverse outcome *in vivo* are established for reference chemicals, including the necessary *in vitro* to *in vivo* extrapolation, the toxicity of many other chemicals acting through the same mode of action could in theory be characterized and predicted based on the responses in the *in vitro* systems and *in silico* models.

A large research initiative ("Safety Evaluation Ultimately Replacing Animal Testing," or SEURAT) is based on this premise (Gocht *et al.*, 2013). The first phase of this program, which is co-funded by the European Commission under its Seventh Framework Programme (FP7) and Cosmetics Europe, spans a 5-year period from 2011 to 2015 and includes six research projects, combining the research efforts of over 70 European universities, public research institutes and companies addressing repeated-dose toxicity in hepatic, cardiac, renal, neuronal, muscle and skin tissues. The strategy involves mode of action analysis to describe how any substance may adversely affect human health and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure for safety and risk assessment.

Where data are available on only one or a limited number of key events and the link to an (adverse) effect has not been sufficiently demonstrated, the data may still be of value in helping to rank and prioritize chemicals, as a basis for additional testing and/or decision-making based on likely relative hazard (e.g., relative potency in modulating sodium channels, endocrine disrupting substance prioritization) (see Case example 5).

Case example 5: Mode of action analysis in prioritizing substances for further testing

There is a great deal of interest in prioritizing chemicals for evaluation of endocrine disruption potential (i.e., how best to focus on those chemicals most likely to cause adverse effects without empirically testing all chemicals of regulatory concern). An expert (QSAR) system was developed to predict estrogen receptor binding affinity, using the mode of action (adverse outcome pathway) knowledge (OECD, 2009; Schmieder *et al.*, 2003, 2004; US EPA, 2009a). This pathway is initiated through direct chemical binding to the estrogen receptor, which could plausibly lead to reproductive impairment. The predictive model was developed based on two *in vitro* assays: using a rainbow trout estrogen receptor competitive binding assay to directly measure the chemical–biological interaction and a trout liver slice assay in which the consequences of estrogen receptor activation or inhibition are measurable as a result of tissue uptake and partitioning of the chemical in the presence of xenobiotic metabolism.

More broadly, consideration of SARs for specific key events known to be involved in the mode of action of representative chemicals with the same structural features would be invaluable in helping to construct chemical categories and would enhance the reliability of read-across (see Case example 6 on pyrethroids and Case example 7 on aniline).

Case example 6: Mode of action in the creation of chemical categories

This example addresses the risk assessment of a new synthetic pyrethroid with the same pesticidal mode of action and insecticidal effects as other members of this structural class of compounds. The critical effect of most pyrethroids is reversible neurotoxicity through interaction with a common target, neuronal sodium channels (reviewed in Soderlund, 2012). This mode of action has been established with confidence, and hence the similarity of the pesticidal mode of action of a new member of this chemical group will provide evidence that the compounds share key events. This can be used to support read-across. The risk assessment of a new pyrethroid could then be based on the assumption that it will share a mode of action with other pyrethroids and its likely relative hazard considered in this manner for a first-tier assessment.

The mode of action involves interaction with neuronal sodium channels (Clark and Symington, 2012; Soderlund, 2012). Hence, interaction with sodium channels is a key event for what is often the critical effect. One could rank existing pyrethroids for their potency in modifying the neuronal sodium channel in a suitably designed *in vitro* system and determine the potency of the new compound in this system (Cao *et al.*, 2011b; McConnell *et al.*, 2012). One would also wish to consider basic toxicokinetic aspects, such as absorption (which could be predicted from lipid solubility) (Hou *et al.*, 2009) and metabolic stability (which could be determined in *in vitro* test systems, such as hepatic microsomal fraction or cultured hepatocytes) (Scollon *et al.*, 2009). This information could be used, either semiquantitatively or with a physiologically based toxicokinetic model (Knaak *et al.*, 2012), to inform the choice of reference point from among those of the compounds for which information is already available.

Hence, by using an established mode of action for a structurally well-defined group of compounds with a common toxicophore, it is possible to inform read-across in the early tiers of a risk assessment. This could be refined by evaluating specific key events *in vitro* and using the resulting information to refine the read-across process. In this way, the results of new *in vitro* approaches can be anchored in relevant outcomes by using existing knowledge and concepts.

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In addition, such information would help in constructing assessment groups for consideration in the risk assessment of combined exposures to multiple chemicals (Cao *et al.*, 2011a).

Case example 7: Use of mode of action analysis to identify critical data needs and testing strategies in read-across

This case example is based on a case study presented at an Organisation for Economic Co-operation and Development (OECD) workshop held in December 2010. It addresses a mode of action related to the formation of methemoglobin and a number of industrial chemicals that are anilines, which vary in the quantity of toxicity data available (European Chemicals Bureau, 2004). It illustrates how the understanding of the mode of action can focus testing and more effectively fill data needs for data-limited compounds.

Aniline induces methemoglobinemia, which, if severe, can result in hemolytic anemia. Hemolytic anemia is a late consequence of methemoglobinemia and a response to the elimination of circulating red blood cells that contain methemoglobin. Aniline is first metabolized in the liver (probably by cytochrome P450 enzymes) to phenylhydroxylamine. It is further oxidized in red cells, most likely to free radical species, via nitrosobenzene. The iron in hemoglobin is oxidized by the free radical species from Fe^{2+} to Fe^{3+}, in which state (i.e., methemoglobin) it cannot bind oxygen. Decreased oxygen results in hypoxia-induced necrosis in tissues that have high oxygen needs. Damaged red blood cells are sequestered by the spleen and are phagocytosed by splenic macrophages, leading to increased red blood cell production by the blood-forming organs, primarily the bone marrow. If the bone marrow cannot keep up with the replacement needs, then extramedullary hematopoiesis occurs as a compensatory response. To determine the potential of the untested anilines to result in hemolytic anemia, *in vitro* testing could be conducted to measure the formation of phenylhydroxylamine and/or methemoglobin.

Thus, the mode of action framework provides a conceptual construct to consider key events at different levels of biological organization plausibly linked to an *in vivo* endpoint of regulatory interest. This allows for the development and use of alternative (*in vitro*) assays to target particular cellular or physiological key events along a specific pathway. Once the mode of action has been established, the key event data can be used for read-across from other chemicals. If a new chemical fits the established mode of action, this existing knowledge can be used to justify a more efficient testing strategy, so not every chemical needs to be evaluated in an *in vivo* test.

Information on mode of action, or on critical key events, can also be invaluable in helping to construct assessment groups for conducting a risk assessment of combined exposure to multiple chemicals (Meek *et al.*, 2011; see Case example 6).

One conclusion from the application of the mode of action framework to information obtained using non-animal methods could be that the data are sufficiently robust to support an established mode of action with a known causal relationship to an (adverse) outcome. Alternatively, it may be possible to conclude that whereas information on one or more key events is missing, provision of information on this data gap would enable a putative mode of action to be assessed with confidence. Finally, the available data may be such that it is not possible to postulate any mode of action with an acceptable degree of confidence.

Increasing numbers of data warehouses comprising substantial amounts of curated information on interspecies and interindividual variability in parameters relevant to many key events are becoming available. These warehouses cover a wide range of species- and individual-specific information, including human demographics, anatomical, physiological, biochemical, clinical chemical and life stage-dependent parameters, genetic, genomic, epigenetic, transcriptomic, proteomic and metabolomic information, phenotypic variation in cellular and physiological functions, and expression levels and activities of enzymes and transporters of xenobiotic disposition. Such information, together with evolving bioinformatics and computational tools, may facilitate quantitative (both deterministic and probabilistic) analyses of variability and more robust uncertainty analyses. These tools may also enable more effective analysis of the frequency with which alterations of key events and pathways are reported in similar studies, within and across animal species, and among humans. Similarly, they may permit a more thorough analysis of dose, exposure durations and response relationships in pathways across studies.

It should be noted that the availability of larger quantities of data on early potential key events to inform mode of action analyses might lend itself to probabilistic assessments and more robust uncertainty analyses.

Discussion and Conclusions

The WHO/IPCS mode of action/human relevance framework has been updated to reflect experience acquired in its application, as well as extending its utility to emerging areas in toxicity testing and non-testing methods. The underlying principles have not changed, but the scope of the framework has been extended to integrate information at different levels of biological organization and to reflect evolving experience in a much broader range of potential applications. These applications are relevant not only to full risk assessment for individual chemicals, but also to evolving methods for priority setting and assessment to meet increasing demands to more efficiently and accurately assess and manage large numbers of substances. They include read-across and assessment of groups of chemicals and combined exposures. The mode of action/species concordance analysis also informs hypothesis-based data generation and research priorities in support of risk assessment, related not only to (adverse) effects but also to therapeutic intervention strategies.

Envisaged broader application is illustrated in an integrative and iterative roadmap to address needs for assessment identified in formal problem formulation, as a basis to tailor the appropriate extent of mode of action/species concordance analysis. The roadmap, problem formulation and framework are iterative in nature, with feedback loops encouraging continuous refinement of fit for purpose testing strategies and risk assessment.

The relationship between mode of action and the more recently defined "adverse outcome pathway" is also clarified: conceptually, the terms are synonymous, with both representing division of the path between exposure and effect into a series of key events (including early molecular initiating events) for both individuals and populations. However, mode of action does

not necessarily imply adversity of effect, as is seemingly implied by the descriptor adverse outcome pathway.

Broader application of the modified mode of action framework is considered in two contexts, including one for which it was originally developed, where the toxicological effects of chemical exposure are known (i.e., when, as a result of problem formulation, there is a desire to perform a mode of action/species concordance analysis for an observed toxicological effect). The outcome of mode of action analysis in this application is acceptance or rejection of a hypothesized mode of action or recommendation for additional targeted research. Various case examples included here illustrate the nature of information required to demonstrate lack of human concordance, the implications of kinetic and dynamic data considered in mode of action analysis for subsequent dose–response analysis and for the design of targeted research studies using new methods (e.g., genomic technologies) and the integration of toxicological and epidemiological data.

The modified framework can also be applied in hypothesizing effects resulting from exposure to a chemical – that is, with information on putative key events in established modes of action from appropriate *in vitro* or *in silico* systems and other lines of evidence to predict and assess the likelihood of a potential mode of action and consequent effects. With the increasing amount of data available from evolving technologies, such as high-throughput and high-content screening assays, QSARs and other computational approaches, it is likely that this latter application of the framework will be of increasing value to the risk assessment community. The considerable experience acquired in the application of the framework in addressing documented (adverse) effects has a meaningful implication to inform the more limited knowledge base in these more predictive applications. This is illustrated in various case examples, including the use of mode of action analysis in prioritizing substances for further testing, in guiding development of more efficient testing strategies and in identifying critical data needs and testing strategies in read-across. In this vein, mode of action considerations should inform further development of research strategies and data generation methods, as well as the development of biomarkers.

The modified Bradford Hill considerations incorporated in framework analysis from its inception are considered a critical element to document, transparently and consistently, weight of evidence for hypothesized modes of action. These considerations have been updated and additionally articulated somewhat here to reflect increasing experience in application for cases where the toxicological outcome of chemical exposure is known. Additional work is also under way to further simplify and delineate application of the modified Bradford Hill considerations in mode of action analysis. This includes additional articulation of the modified Bradford Hill considerations for weight of evidence as a basis to contribute to common understanding, rank ordering of their importance as well as provision of examples of what might constitute strong versus weak evidence for each, based on acquired experience in mode of action analysis (Meek ME, Palermo CM, Bachman AM, North CM, Lewis RJ, submitted).

A template for extension of the concordance table in the original framework to dose–response analysis is also included, as is one for comparative consideration of weight of evidence for various modes of action based on the modified Bradford Hill considerations. Clear and transparent documentation of uncertainties at each stage of the mode of action analysis is also emphasized, with the objective of being as quantitative as possible regarding the likelihood of a hypothesized mode of action being operative in humans. Additional work to delineate more specifically the appropriate form and content of uncertainty analysis is strongly recommended, consistent with objectives and content of ongoing initiatives in this area.

Experience in mode of action analyses for documented (adverse) effects in human health risk assessment is informative in consideration of weight of evidence for hypothesized effects (referenced as adverse outcome pathways by OECD, 2012), based on early key or molecular initiating events. Based on this experience, development of proof of concept for application of the modified Bradford Hill considerations in more predictive application is strongly recommended. This is particularly important, in view of their significant reliance on demonstration of the essentiality of key events and concordance of dose–response relationships and temporality between early and late key events, information that is often lacking in the more predictive application that is envisaged. Additional collaboration between the health risk and ecological communities in this context is also recommended as a basis to draw on collective experience to increase common understanding and to develop communication and uptake strategies.

In conclusion, the modified framework and accompanying roadmap and case examples are expected to contribute to improving transparency in explicitly addressing weight of evidence considerations in mode of action and species concordance analyses based on both conventional data sources and evolving methods. The broader application envisaged here emphasizes the importance of interaction among the risk assessment, risk management and research communities, as a basis to transition to consideration of data from different levels of biological organization in fit for purpose mode of action analysis (e.g., prioritization vs. full assessment), while also highlighting the need to anchor data from evolving technologies and research. Development of the modified mode of action framework has also highlighted the conceptually identical mode of action and adverse outcome pathway and the resulting need for the research and environmental and human health risk assessment communities to move forward together to develop rigorous, efficient and transparent methodologies to meet increasingly progressive mandates to test and assess, more efficiently and more effectively, much larger numbers of chemical substances in commerce.

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Conflict of Interest

The authors did not report any conflicts of interest.

References

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* **29**: 730–741.

Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek ME, Vickers C, Willcocks D, Farland W. 2006. IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**: 781–792.

Boobis AR, Doe JE, Heinrich-Hirsch B, Meek ME, Munn S, Ruchirawat M, Schlatter J, Seed J, Vickers C. 2008. IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit. Rev. Toxicol.* **38**: 87–96.

Boobis AR, Daston GF, Preston RJ, Olin SS. 2009. Application of key events analysis to chemical carcinogens and noncarcinogens. *Crit. Rev. Food Sci. Nutr.* **49**: 690–707.

Cao Z, Shafer TJ, Crofton KM, Gennings C, Murray TF. 2011a. Additivity of pyrethroid actions on sodium influx in cerebrocortical neurons in primary culture. *Environ. Health Perspect.* **119**: 1239–1246.

Cao Z, Shafer, TJ, Murray TF. 2011b. Mechanisms of pyrethroid insecticide-induced stimulation of calcium influx in neocortical neurons. *J. Pharmacol. Exp. Ther.* **336**: 197–205.

Carmichael N, Bausen M, Boobis AR, Cohen SM, Embry M, Fruijtier-Pölloth C, Greim H, Lewis R, Meek ME, Mellor H, Vickers C, Doe J. 2011. Using mode of action information to improve regulatory decision-making: an ECETOC/ILSI RF/HESI workshop overview. *Crit. Rev. Toxicol.* **41**: 175–186.

Clark JM, Symington SB. 2012. Advances in the mode of action of pyrethroids. *Top. Curr. Chem.* **314**: 49–72.

Cohen SM, Boobis AR, Meek ME, Preston RJ, McGregor D. 2006a. 4-Aminobiphenyl and DNA reactivity: case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**: 803–819.

Cohen SM, Arnold LL, Eldan M, Lewis AS, Beck BD. 2006b. Methylated arsenicals: the implications of metabolism and carcinogenicity studies in rodents to human risk assessment. *Crit. Rev. Toxicol.* **36**: 99–133.

Cohen SM, Ohnishi T, Arnold LL, Le SC. 2007. Arsenic-induced bladder cancer in an animal model. *Toxicol. Appl. Pharmacol.* **222**: 258–263.

Council of Labor Affairs, Taiwan. 2012. Existing Chemical Substance Inventory. Chemical Substance Nomination & Notification, Council of Labor Affairs, Executive Yuan, Taiwan. Available at: http://csnn.cla.gov.tw/content/englishHome.aspx (accessed 28 September 2012).

Dellarco VL, McGregor D, Berry SC, Cohen SM, Boobis AR. 2006. Thiazopyr and thyroid disruption: case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action. *Crit. Rev. Toxicol.* **36**: 793–801.

Dellarco V, Henry T, Sayre P, Seed J, Bradbury S. 2010. Meeting the common needs of a more effective and efficient testing and assessment paradigm for chemical risk management. *J. Toxicol. Environ. Health B Crit. Rev.* **13**(2–4): 347–360.

European Chemicals Bureau. 2004. European Union Risk Assessment Report: Aniline. Office for Official Publications of the European Communities, Luxembourg. Available at: http://esis.jrc.ec.europa.eu/doc/risk_assessment/REPORT/anilinereport049.pdf (accessed 28 September 2012).

European Commission. 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC,93/67/EEC, 93/105/EC and 2000/21/EC. Off. J. Eur. Union L396, 1–849. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:396:0001:0849:EN:PDF (accessed 28 September 2012).

Gocht T, Schwarz M, Berggren E, Whelan M. 2013. SEURAT-1: Development of a Research Strategy for the Replacement of *In Vivo* Repeated Dose Systemic Toxicity Testing. Available at: http://www.alttox.org/ttrc/eu/way-forward/gocht-schwarz-berggren-whelan/#1 (accessed 13 September 2013).

Hill AB. 1965. The environment and disease: association or causation? *Proc. R. Soc. Med.* **58**: 295–300.

Hou T, Li Y, Zhang W, Wang J. 2009. Recent developments of *in silico* predictions of intestinal absorption and oral bioavailability. *Comb. Chem. High Throughput Screen.* **12**: 497–506.

Hughes K, Paterson J, Meek ME. 2009. Tools for the prioritization of substances on the Domestic Substances List in Canada on the basis of hazard. *Regul. Toxicol. Pharmacol.* **55**: 382–393.

Julien E, Boobis AR, Olin SS, The ILSI Research Foundation Threshold Working Group. 2009. The key events dose–response framework: a cross-disciplinary mode-of-action based approach to examining dose–response and thresholds. *Crit. Rev. Food Sci. Nutr.* **49**: 682–689.

Knaak JB, Dary CC, Zhang X, Gerlach RW, Tornero-Velez R, Chang DT, Goldsmith R, Blancato JN. 2012. Parameters for pyrethroid insecticide QSAR and PBPK/PD models for human risk assessment. *Rev. Environ. Contam. Toxicol.* **219**: 1–114.

Lowell Center for Sustainable Production. 2012. Chemicals Policy: Other Nations [Internet]. Chemicals Policy & Science Initiative, Lowell Center for Sustainable Production, University of Massachusetts Lowell, Lowell, Massachusetts. Available at: http://www.chemicalspolicy.org/chemicalspolicy.othernations.php (accessed 28 September 2012).

McConnell ER, McClain MA, Ross J, Lefew WR, Shafer TJ. 2012. Evaluation of multi-well microelectrode arrays for neurotoxicity screening using a chemical training set. *Neurotoxicology* **33**: 1048–1057.

McGregor D, Bolt H, Cogliano V, Richter-Reichhelm HB. 2006. Formaldehyde and glutaraldehyde and nasal cytotoxicity: case study within the context of the 2006 IPCS human framework for the analysis of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**: 821–835.

Meek ME. 2008. Recent developments in frameworks to consider human relevance of hypothesized modes of action for tumours in animals. *Environ. Mol. Mutagen.* **49**: 110–116.

Meek ME. 2009. Mode of Action Frameworks in Toxicity Testing and Chemical Risk Assessment. PhD dissertation, Institute for Risk Assessment Sciences, Utrecht University, the Netherlands.

Meek ME, Armstrong VC. 2007. The assessment and management of industrial chemicals in Canada. In *Risk Assessment of Chemicals: An Introduction*, Van Leeuwen K, Vermeire T (eds). 2nd edn. Kluwer Academic Publishers: Dordrecht, the Netherlands; 591–621.

Meek ME, Klaunig JE. 2010. Proposed mode of action of benzene-induced leukemia: interpreting available data and identifying critical data gaps for risk assessment. *Chem. Biol. Interact.* **184**: 279–285.

Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD, Longfellow DG, Pastoor T, Seed J, Patton DE. 2003. A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **33**: 591–653.

Meek ME, Berry C, Boobis AR, Cohen SM, Hartley M, Munn S, Olin S, Schlatter V, Vickers C. 2008. Letter to the editor re: Guyton, Kathryn Z, Barone, Stanley, Jr, Brown, Rebecca C, Euling, Susan Y, Jinot, Jennifer, Makris, Susan (2008). Mode of action frameworks: a critical analysis. *J. Toxicol. Environ. Health B* **11**: 681–685.

Meek ME, Boobis AR, Crofton KM, Heinemeyer G, Raaij MV, Vickers C. 2011. Risk assessment of combined exposure to multiple chemicals: a WHO/IPCS framework. *Regul. Toxicol. Pharmacol.* **60**(2): S1–S14.

Meek ME, Palermo CM, Bachman AM, North CM, Lewis RJ. n.d. Submitted. Mode of action human relevance (MOA/HR) framework – Evolution of the modified Bradford Hill considerations and comparative analysis of weight of evidence. *Appl. Toxicol.*

[NRC] National Research Council. 2007. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. National Academy Press: Washington, DC.

[OECD] Organisation for Economic Co-operation and Development. 2009. Report of the Expert Consultation to Evaluate an Estrogen Receptor Binding Affinity Model for Hazard Identification. Environment Directorate, OECD, Paris. Available at: http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2009)33&doclanguage=en (accessed 28 September 2012).

[OECD] Organisation for Economic Co-operation and Development. 2012. Proposal for a Template, and Guidance on Developing and Assessing the Completeness of Adverse Outcome Pathways. OECD, Paris. Available at: http://www.oecd.org/chemicalsafety/testingofchemicals/49963554.pdf (accessed 28 September 2012).

Schmieder P, Mekenyan O, Bradbury S, Veith G. 2003. QSAR prioritization of chemical inventories for endocrine disruptor testing. *Pure Appl. Chem.* **75**: 2389–2396.

Schmieder PK, Tapper MA, Denny JS, Kolanczyk RC, Sheedy BR, Henry TR, Veith GD. 2004. Use of trout liver slices to enhance mechanistic

interpretation of estrogen receptor binding for cost-effective prioritization of chemicals within large inventories. *Environ. Sci. Technol.* **38**(23): 6333–6342.

Scollon EJ, Starr JM, Godin SJ, DeVito MJ, Hughes MF. 2009. *In vitro* metabolism of pyrethroid pesticides by rat and human hepatic microsomes and cytochrome p450 isoforms. *Drug Metab. Dispos.* **37**: 221–228.

Seed J, Carney EW, Corley RA, Crofton KM, DeSesso JM, Foster PM, Kavlock R, Kimmel G, Klaunig J, Meek ME, Preston RJ, Slikker W, Jr, Tabacova S, Williams GM, Wiltse J, Zoeller RT, Fenner-Crisp P, Patton DE. 2005. Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Crit. Rev. Toxicol.* **35**: 664–672.

Soderlund DM. 2012. Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. *Arch. Toxicol.* **86**: 165–181.

Sonich-Mullin C, Fielder R, Wiltse J, Baetcke K, Dempsey J, Fenner-Crisp P, Grant D, Hartley M, Knaap A, Kroese D, Mangelsdorf I, Meek E, Rice JM, Younes M, International Programme on Chemical Safety. 2001. IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul. Toxicol. Pharmacol.* **34**: 146–152.

[US EPA] US Environmental Protection Agency. 1996. Proposed guidelines for carcinogen risk assessment. *Fed. Reg.* **61**: 17960–18011.

[US EPA] US Environmental Protection Agency. 2005a. Guidelines for Carcinogen Risk Assessment. US EPA, Washington, DC. Available at: http://www.epa.gov/cancerguidelines/

[US EPA] US Environmental Protection Agency. 2005b. Science Issue Paper: Mode of Carcinogenic Action for Cacodylic Acid (Dimethylarsinic Acid, DMA^V) and Recommendations for Dose Response Extrapolation. Health Effects Division, Office of Pesticide Programs, US EPA, Washington, DC.

[US EPA] US Environmental Protection Agency. 2009a. An Effects-based Expert System to Predict Estrogen Receptor Binding Affinity for Food Use Inert Ingredients and Antimicrobial Pesticides: Application in a Prioritization Scheme for Endocrine Disrupting Screening [Draft]. Office of Pesticide Programs, US EPA, Washington, DC. Available at: http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2009-0322-0002 (accessed 28 September 2009).

[US EPA] US Environmental Protection Agency. 2009b. Integrated Science Assessment for Particulate Matter (Final Report). EPA/600/R-08/139F. US EPA, Washington, DC, pp. 5-1–5-23.

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Mode of action human relevance (species concordance) framework: Evolution of the Bradford Hill considerations and comparative analysis of weight of evidence

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ABSTRACT: The mode of action human relevance (MOA/HR) framework increases transparency in systematically considering data on MOA for end (adverse) effects and their relevance to humans. This framework continues to evolve as experience increases in its application. Though the MOA/HR framework is not designed to address the question of "how much information is enough" to support a hypothesized MOA in animals or its relevance to humans, its organizing construct has potential value in considering relative weight of evidence (WOE) among different cases and hypothesized MOA(s). This context is explored based on MOA analyses in published assessments to illustrate the relative extent of supporting data and their implications for dose–response analysis and involved comparisons for chemical assessments on trichloropropane, and carbon tetrachloride with several hypothesized MOA(s) for cancer. The WOE for each hypothesized MOA was summarized in narrative tables based on comparison and contrast of the extent and nature of the supporting database versus potentially inconsistent or missing information. The comparison was based on evolved Bradford Hill considerations rank ordered to reflect their relative contribution to WOE determinations of MOA taking into account increasing experience in their application internationally. This clarification of considerations for WOE determinations as a basis for comparative analysis is anticipated to contribute to increasing consistency in the application of MOA/HR analysis and potentially, transparency in separating science judgment from public policy considerations in regulatory risk assessment. Copyright © 2014. The Authors. Journal of Applied Toxicology Published by John Wiley & Sons Ltd.

Keywords: human relevance framework; mode of action; weight of evidence; key events; evolved Bradford Hill considerations

Introduction

The mode of action/human relevance (MOA/HR) framework is an analytical framework designed to increase transparency in the systematic consideration of the weight of evidence (WOE) of hypothesized MOA(s) for critical effects and their relevance to humans. It was developed in initiatives of the International Life Sciences Institute Risk Sciences Institute (ILSI RSI) and the International Programme on Chemical Safety (IPCS) and derives from earlier work on MOA by the US Environmental Protection Agency (USEPA) and IPCS (Sonich-Mullin *et al.*, 2001).

The development and evolution of the IPCS ILSI RSI MOA/HR framework, which has involved large numbers of scientists internationally, is described in several publications (Boobis *et al.*, 2006, 2008; Meek, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005). Potential application in a broader range of relevant contexts has been considered more recently (Carmichael *et al.*, 2011; Meek and Klaunig, 2010). The framework has been illustrated by an increasing number of case studies ($n = 30$, currently), and is widely adopted in international and national guidance and assessments (Meek *et al.*, 2008), including those of the USEPA (Dellarco and Baetcke, 2005; Manibusan *et al.*, 2007; SAB, 1999, 2007; SAP, 2000; USEPA, 2005a). Building on this collective experience, the framework has been updated recently, to address uncertainty additionally and to extend its utility to emerging areas in toxicity testing and non-testing methods. The update includes incorporation within a roadmap, encouraging continuous refinement of fit-for-purpose testing strategies and risk assessment (Meek *et al.*, 2014).

In addition to increasing transparency through structured articulation of the evidence and uncertainties upon which conclusions are based, MOA/HR analysis also contributes to the transparent assimilation of all available data in both a risk assessment and research context. This is important because it facilitates identification of critical data needs and contributes to transparency in the separation of science judgment (i.e., weighting of options based on systematic consideration of available scientific support) from public health protection policy, the latter

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sometimes involving embedded conservatism to increase public health protection.

Though the MOA/HR framework is not designed to address the question of "how much information is enough" to support a hypothesized MOA in animals or its relevance to humans, its organizing construct has value in considering relative WOE among different cases and hypothesized MOAs. Comparative WOE evaluation for MOA/HR analysis is illustrated as a basis to increase common understanding of the nature of transparency required to document the relative degree of confidence in supporting data for hypothesized MOAs. To demonstrate this approach, WOE for MOA/HR analysis in two published assessments (i.e., carbon tetrachloride and 1,2,3-trichloropropane [TCP]) (USEPA, 2009, 2010) is comparatively considered in the context of evolved Bradford Hill (B/H) considerations introduced here to promote better common understanding and consistency in use. The focus here is not on the conclusions of the assessments but rather, the utility of comparative analysis for WOE evaluation in MOA/HR analysis. These cases were specifically selected to exemplify varying degrees of WOE for several hypothesized MOA.

Methods And Results

Details of the updated MOA/HR framework are available elsewhere (Meek *et al.*, 2014). Briefly, the WOE for a hypothesized MOA in animals is assessed based on considerations modified from those proposed by Bradford Hill (Hill, 1965) for assessment of causality in epidemiological studies. HR or species concordance is then systematically considered, taking into account more generic information such as anatomical, physiological and biochemical variations. If the WOE for the hypothesized MOA is sufficient and relevant to humans, implications for dose–response in humans are then considered in the context of kinetic and dynamic data. Delineation of the degree of confidence in the WOE for hypothesized MOAs is critical, as is the delination of critical research needs.

Establishing support for or rejection of a hypothesized MOA provides the foundation for subsequent considerations of dose–response, HR and estimates of risk. It involves (1) delineation of key events leading to the end (adverse) effect in a hypothesized MOA and (2) evaluation of all of the data to consider the extent of the supporting WOE for the hypothesized MOA. Importantly, if alternative MOA(s) are supported, these are evaluated with equal rigor in separate MOA/HR framework analyses. Ultimately, depending upon the application, there may be a need to draw a conclusion on the sufficiency of data supporting a MOA, to assess different risk management options. The comparative analysis of WOE was developed as a basis for increasing common understanding of the nature of transparency required to document the degree of confidence in the sufficiency of supporting data for hypothesized (potentially competing) MOAs.

A template for WOE analysis of MOA based on the evolved B/H considerations is presented in Table 1. In this approach, supporting data, inconsistent data and missing information are evaluated and tabulated in the context of the evolved B/H considerations presented here. The data in this table are considered in totality to assess the WOE for a MOA. In addition, the evidence can be used in a comparative manner to gain perspective on the relative degree of confidence that a hypothesized MOA is operative, based on the extent of supporting WOE compared to that for another postulated MOA for the same chemical or for the same MOA for other chemicals.

As illustrated in Table 1, WOE analysis is heavily dependent on the B/H considerations. Previous iterations of modified B/H considerations have been applied inconsistently in MOA/HR analyses, which may be attributable in large measure to the availability of only relatively general, early guidance in this area (USEPA, 2005b; Sonich-Mullin *et al.*, 2001). Some of the considerations have been misinterpreted due to a lack of common understanding of their appropriate level of application to MOA data in a WOE context; i.e., in overall data synthesis and evaluation of sufficiency of evidence to support a MOA decision versus the initial phase of systematic review (i.e., data selection and individual study review). Table 2 summarizes the variation in definitions of the B/H considerations in MOA analysis, which may also have contributed to inconsistency in application.

Evolved B/H considerations have been proposed and clarified here through delineation of the specific aspects addressed by each, as framed by a series of questions (captured below and summarized in Table 3). These questions build on those presented in Meek *et al.* (2014), based on additional experience in considering transparency in existing assessments as a basis to document comparative WOE. These evolved B/H considerations are proposed, then, not only as a basis to increase consistency in making WOE determinations for hypothesized MOA(s), but also to

Table 1. Template for weight of evidence based on evolved Bradford Hill considerations

Evolved Bradford Hill Considerations	Supporting Data	Inconsistent Data	Missing Data
1. Biological Concordance			
2. Essentiality of Key events			
3. Concordance of Empirical Observations among Key Events	Dose–response Temporality Incidence		
4. Consistency			
5. Analogy			

For a postulated mode of action, supporting data, inconsistent data and missing data are tabulated in the context of the evolved Bradford Hill considerations. Input in the supporting and inconsistent columns captures only what has been observed. Input in the missing column includes only that which is technically feasible and that is important for informing the mode of action. Cells are left blank in instances where data do not exist or are inadequate for evaluation. A brief narrative should accompany this table to describe the overall determination as to whether the data support or refute the hypothesis.

Table 2. Definition of the Bradford Hill considerations for application in mode of action analysis

Bradford Hill Considerations (Hill, 1965)	IPCS MOA/HR Framework (Boobis *et al.*, 2006; 2008; Sonich-Mullin *et al.*, 2001)	EPA Cancer Guidelines (USEPA, 2005b)	Evolved Bradford Hill considerations
Strength Strength of the association between suspected cause and observation.	**Strength** Unclearly defined. Considered together with specificity and consistency.	**Strength** The finding of large risks increases confi dence the association is not due to chance.	**N/A** Not considered applicable for evaluating MOA data.
Consistency Repeatability of an association by different persons, in different places, circumstances and times.	**Consistency** Repeatability of the key events in different studies. Considered together with strength and specificity.	**Consistency** Pattern of elevated risk observed across several independent studies.	**Consistency** Is the pattern of effects across species/ strains/ organs/test systems what would be expected?
Specificity The association is limited to a specific population and to particular sites and types of disease.	**Specificity** Stop/recovery studies show an absence or reduction of toxicity when a key event is blocked or reduced. Considered together with strength and consistency.	**Specificity** One cause associated with a single effect or disease.	**Essentiality of key events** Is the sequence of events reversible if dosing is stopped or a key event prevented?
Temporality The exposure occurs before the effect.	**Temporal association** Key events should be observable before toxicity is apparent.	**Temporal relationship** When exposure is known to precede development of the disease.	**Temporal concordance** Are the key events observed in hypothesized order?
Biological gradient Risk of disease increases with increasing exposure.	**Dose–response relationship** The dose–response for key events parallel the dose–response for the toxic effect. Increases in incidence of a key event correlate with increase in incidence of later key events.	**Biological gradient** Increasing effects associated with greater exposure.	**Dose–response concordance** Are the key events observed at doses below or similar to those associated with the end (adverse) effect?
Plausibility Biological knowledge supports suspected causation.	**Biological plausibility and coherence** Consistent with current understanding of biology. Considered together with coherence.	**Biological plausibility** Consistency with data from experiments or other sources demonstrating biological plausibility.	**Biological concordance** Does the hypothesized MOA conflict with broader biological knowledge? How well established is the MOA in the wider biological database?
Coherence The association agrees with the generally known facts of the history and biology of the disease.	**Coherence** Consistency with what is known specifically about the overall biological effects of the substance. Considered together with biological plausibility.	**Coherence** Information supporting cause and effect from other lines of evidence (i.e., animal bioassays, toxicokinetic studies and short-term studies).	**N/A** Not considered applicable for evaluating MOA data
Experiment Experimental evidence alters the frequency of associated events.	**N/A** Has not been mentioned in recent publications on the MOA/HR framework.	**Experimental evidence** when a change of exposure in a human popu lation brings about a change in disease.	**N/A** Not considered applicable for evaluating MOA data.

(Continues)

Table 2. *(Continued)*

Bradford Hill Considerations (Hill, 1965)	IPCS MOA/HR Framework (Boobis *et al.*, 2006; 2008; Sonich-Mullin *et al.*, 2001)	EPA Cancer Guidelines (USEPA, 2005b)	Evolved Bradford Hill considerations
Analogy Information for a similar but different association supports causation. **N/A**	**N/A** Has not been mentioned in recent publications on the HR/MOA framework. **N/A** Considered as part of dose–response relationship definition.	**Analogy** Insight gained from structure activity relationships and information on structural analogues. **N/A**	**Analogy** Would the MOA be anticipated based on broader chemical specific knowledge? **Incidence concordance** Is the occurrence of the end (adverse) effect less than that for preceding key events?

HR, human relevance; MOA, mode of action.

promote consistency in their application based on accumulating experience internationally.

The evolved B/H considerations are described in more detail below. These considerations appear in rank order based on their appropriate weighting of relative contribution to WOE determinations for hypothesized MOA(s), with those listed first contributing most significantly. Examples for evaluating weak to strong evidence for each evolved B/H consideration are also discussed.

Biological Concordance

- Does the hypothesized MOA conflict with broader biological knowledge?
- How well established is the MOA?

Evidence for a hypothesized MOA must satisfy the consideration of biological concordance. If available data on the hypothesized MOA are at odds with biological understanding, the hypothesis does not constitute a reasonable option for consideration. For instance, if a hypothesized early key event cannot conceivably lead to a subsequent hypothesized key or end event, it need not be considered.

The extent of evidence for biological concordance would be considered stronger, for example, if the hypothesized MOA has been well documented for a broad range of chemicals, and weaker if the hypothesized MOA is conceivable based on limited data or it has been hypothesized based simply on the possibility that none of the key events are at odds with biological understanding.

Essentiality of Key Events

- Is the sequence of events reversible if dosing is stopped or a key event prevented (i.e., counterfactual evidence)?

The extent of counterfactual evidence (i.e., experimental support for the necessity of a key event) is one of the principal determinants of WOE for a hypothesized MOA (Borgert *et al.*, 2011). For example, experimental evidence in animal models that lack a key metabolic pathway (e.g., knock out animal models) and fail to develop the end (adverse) effect would support essentiality of a key event. Similarly, if following cessation of repeated exposure for various periods, effects are reversible (i.e., late key events and/or the end (adverse) effect is prevented), this constitutes relatively strong evidence that key events are causal.

It is important to note that by its nature, counterfactual evidence typically addresses the necessity of an individual key event in a hypothesized MOA. Therefore, it may not always be helpful for discerning between two possible MOAs that share a key event. For example, if a chemical requires metabolic activation to be carcinogenic, a negative result in a 2-year cancer bioassay in an animal model null for the necessary activating enzyme supports that metabolism is necessary for carcinogenesis but is not helpful for differentiating between a MOA involving metabolic activation followed by direct DNA damage versus a MOA involving metabolic activation followed by cytotoxicity and regenerative proliferation.

Support for the essentiality of key events is considered stronger when there is direct counterfactual evidence supporting multiple key events in the hypothesized MOA. Evidence is considered weaker when evidence involves indirect measures for key events (i.e., the key event is inferred from the actual measured endpoint)

Table 3. Proposed changes to the Bradford Hill considerations and guidance for interpretation to improve application in the MOA/HR framework[a]

Evolved Bradford Hill considerations	Defining questions	Evidence for evaluating degree of support for the mode of action	
		Stronger	Weaker
1. Biological Concordance (replaces biological plausibility & coherence)	Does the hypothesized MOA conflict with broader biological knowledge? How well established is the MOA?	MOA is well established in scientific knowledge and/or completely consistent with established biological understanding.	MOA is contrary to well established biological understanding. MOA requires biological processes that are novel or poorly established.
2. Essentiality of Key Events (replaces strength, and specificity)	Is the sequence of events reversible if dosing is stopped or a key event prevented?	Counterfactual evidence to support key events (e.g., absence/reduction of later events when an earlier key event is blocked or diminished).	Data on reversibility only, indirect evidence only for key events or limited data available to assess.
3. Concordance of Empirical Observations among Key events (encompasses dose response and temporal concordance and beyond)	Dose–response: Are the key events observed at doses below or similar to those associated with end (adverse) effect? Temporality: Are the key events observed in hypothesized order? Incidence: Is the occurrence of the end (adverse) effect less than that for the preceding key events?	Dose–response and temporality: expected pattern of temporal and dose–response relationships based on robust database (multiple studies with examination of key events at interim time periods and at least 3 doses). Incidence: incidence of early key events is greater than end (adverse) effect.	All key events occur at all dose levels and all time points and/or limited data available to assess (e.g., inadequate dose spacing, missing key time periods for effect development, or failure to assess incidence at early time points). Incidence of early key events is lower than the end (adverse) effect and/or limited data available to assess.
4. Consistency (among different biological contexts)	Is the pattern of observations across species/strains/organs/test systems what would be expected based on the hypothesized MOA?	Pattern of effects are what would be expected across species, strains, organs and/or test systems.	Significantly inconsistent pattern of effects or limited data available to assess (e.g., effect only observed in a single rat strain).
5. Analogy (consistency across chemicals)	Would the MOA be anticipated based on broader chemical specific knowledge (e.g., the chemical is a member of a category for which related chemicals have known or strongly suspected MOA)?	Observations are consistent with those for other (related) chemicals having well defined MOA.	Pattern of effects for other (related) chemicals is distinctly different. Insufficient data to evaluate whether chemical behaves like related chemicals with similar proposed MOA.

MOA, mode of action.

[a]Evolution of the Bradford Hill (B/H) considerations for improved fit-for-purpose in the evaluation of sufficiency of data to support a hypothesized MOA. The evolved B/H considerations are rank ordered based on their appropriate weighting of relative contribution to weight of evidence determinations for hypothesized MOA(s), with those listed at the top contributing most significantly.

or non-specific inhibition of key events. For example, for a MOA hypothesized to involve binding to a receptor, demonstrating an end (adverse) effect is prevented by knocking-out or downregulating expression of the receptor is stronger than counterfactual evidence using a non-specific inhibitor.

Concordance of Empirical Observation Among Key Events

Concordance of empirical observations contributes considerably to the WOE for hypothesized MOA(s). Specifically, concordance of dose–response, temporality and incidence are key considerations. Each of these is addressed separately below. While not weighted as heavily as biological concordance and essentiality of key events, concordance of empirical observation across dose–response, temporality and incidence contributes significantly to WOE. Relationships and outliers should be carefully evaluated to understand whether the WOE strongly supports or is discordant with the hypothesized MOA, including consideration of cohesiveness across all three aspects of empirical observation.

Concordance of Dose–response Relationships Among Key Events

- Are the key events observed at doses below or similar to those associated with the end (adverse) effect?

In past MOA analyses, assessment of dose–response has sometimes been misinterpreted as simply addressing the question: "Is there evidence of a dose–response relationship for key events and/or the end (adverse) effect?" While this question is relevant to hazard characterization, it does not address dose–response concordance in relation to the WOE for a hypothesized MOA. Rather, the latter addresses the consistency of observed dose–response relationships among key and end (adverse) effects, as framed explicitly in the question above.

The hypothesized MOA is not supported in scenarios for which there is evidence that early key events occur only at higher doses than the end (adverse) effect. For example, a hypothesized receptor-based MOA is not supported by evidence indicating that receptor binding occurs only at doses well above those that cause frank liver injury, though it is important to consider if this might be a function of dose spacing in the relevant studies. Benchmark dose analyses for the dose–response relationships in key and end events are the most appropriate measure for consideration of their concordance, as they provide for direct comparison of comparable doses associated with a specified increase in each of the key events and/or end (adverse) effects and normalize for variations in dose spacing and group sizes in different studies.

Examination of the pattern of dose–response relationships is particularly important in considering the degree of support for hypothesized mutagenic MOAs (i.e., where mutation is an early and influential key event). For example, observation of a mutagenic response at high (cytotoxic) doses in genotoxicity assays is supportive of hypothesized MOAs where mutation is a secondary consequence of increased proliferative response resulting from tissue damage.

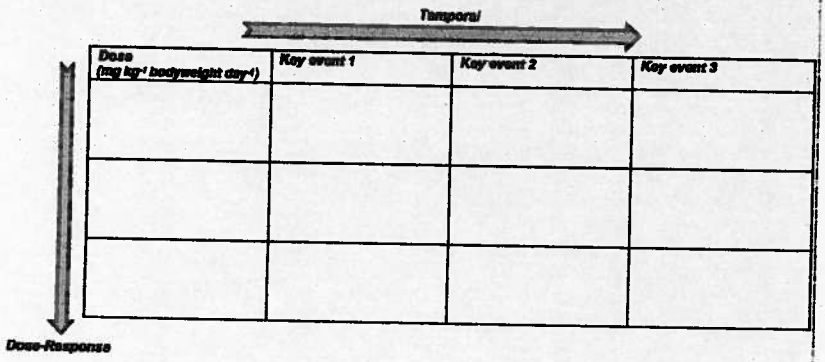
Concordance of Temporality (Time) Among Key Events

- Are the key events observed in hypothesized order?

Temporal concordance refers to the observation of key events in sequential order as described in the hypothesized MOA. In other words, earlier key events should be observed to precede later key events and the late (adverse) effect. Stronger evidence for temporal concordance is obtained when key events at interim time points demonstrate the hypothesized order (either in a single robust study or across multiple studies). Such evidence can often be acquired in studies examining the reversibility of key events and end (adverse) effects following various periods of exposure. Weaker evidence occurs when temporal data on key events are missing.

The template presented in Table 4 is often helpful in determining the extent to which evidence fulfills consideration of dose–response and temporal concordance in WOE analysis for MOA. If the hypothesized MOA is supported, the table should fill diagonally from the top left-hand corner to the bottom right-hand corner. This "pattern" supports a continuum of the relationship between early key events occurring at lower doses than late key events and outcome. Evidence of dose–response and temporal concordance is, for example, weaker if all key events occur at all dose levels and time points. Evidence is stronger, for example, if there is a reasonable range of studies of different durations with a minimum of three dose levels each and the "pattern" of results in this table (Table 4) is as described above.

Table 4. Dose–response and temporal concordance analysis template



Source: Meek and Klaunig (2010).

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Concordance of Incidence Between Key Events and End (Adverse) Effects

- Is the occurrence of the end (adverse) effect less than that for the preceding key events?

Clear evidence of the concordance of the incidence of the end (adverse) effect with that for early hypothesized key events is influential in contributing to WOE for hypothesized MOA(s). The incidence of hypothesized early key events should be greater than that for later key events and the (adverse) outcome, consistent with the important biological underpinning that key events are essential but not necessarily sufficient, to induce the relevant end (adverse) effect. For example, the hypothesis that cytotoxicity followed by regenerative proliferation are key events in the induction of specific tumors would be supported by the observation that the incidence of the former (cytotoxicity/regenerative proliferation) is greater than that for the latter (tumors) at a similar dose. "Incidence" here refers to the occurrence of a lesion of defined severity for each of the key and end events. It should be noted that a 1: 1 correlation of the incidence of early and late key events is not anticipated; lack of evidence for a 1: 1 correlation does not detract from contribution to the overall WOE. Consistent with the essentiality (but not necessarily sufficiency) of key events, lack of 1: 1 concordance is not unexpected, being a function of biological variability; i.e., lesions will not have progressed to the end (adverse) effect in all animals at the termination of exposure.

Consistency

- Is the pattern of observations across species/strains/organs/test systems what would be expected based on the hypothesized MOA?

Evidence of internal consistency within the collective data set for a chemical contributes to increased confidence in the WOE supporting a MOA. For example, if the initial hypothesized key event is oxidative metabolism to a reactive intermediate, are the target tissues and organs those which would be expected based on knowledge of distribution of the relevant metabolic enzyme? Evidence of consistency is stronger if the pattern of species-, strain- and sex-related variations in response is what would be expected based on known differences in metabolic profiles (e.g., extent and rate of metabolism to the putatively toxic entity). Evidence is weaker if there is either significant inconsistency in the expected pattern of the collective data based on the hypothesized MOA (e.g.,. the effect or result is only demonstrated in a single rat strain when data are available for multiple strains, for all of whom metabolizing capacity for the relevant pathway is anticipated to be similar) or when there are limited data available to assess this aspect.

Analogy

- Would the MOA be anticipated based on broader chemical specific knowledge?

Convincing evidence that the hypothesized MOA is operative for a broad range of chemically similar substances also contributes significantly to WOE. For example, consider the case where reductive metabolism for chemically similar substances is associated with a particular pattern of observations leading to the end (adverse) effect. If the pattern of observations for a related chemical is distinctly different, the evidence is weaker that these effects are produced by a similar MOA. On the other hand, if there is an extensive database illustrating that the MOA of interest is operative and leads to similar end (adverse) effects for several closely structurally related chemicals as identified, for example, by (quantitative) structure–activity modeling, evidence is stronger.

The rank order of the B/H considerations suggested above reflects their relative contribution to WOE determinations of MOA and is based on evolving experience internationally. In essence, data that conflicts with a broader biological understanding ranked highly here may be grounds for considering the available supporting data as inconsistent with the hypothesized MOA, whereas lack of concordance of some empirical data is often due to variations in, for example, dose spacing or administered doses in various studies and based on careful evaluation, would not detract meaningfully from the supporting database. In assessing the totality of the WOE, it is helpful to systematically take into account all of the considerations presented here as a basis to contribute to transparency in decision making. Such assessment benefits most from multidisciplinary input from both the relevant research and risk assessment communities. However, there is no minimum number of these evolved B/H considerations that must be met to determine sufficiency and/or associated confidence but rather, in their careful, systematic, more transparent and consistent consideration, cohesiveness (or not) of the supporting data becomes evident. It is also important to recognize that while some of the evolved B/H considerations may address the association of just one key event to the end event (e.g., essentiality of key events) the WOE determination is based on consideration of the interdependence of the key and end events in the hypothesized MOA.

Comparative Weight of Evidence Case Studies

To illustrate the utility of the comparative WOE approach, assessments for two chemicals (USEPA, 2009, 2010) were selected as case studies (i.e., carbon tetrachloride and TCP). The assessment of carbon tetrachloride drew on a previous evaluation of the US EPA (Manibusan *et al.*, 2007), though the conclusions varied. These assessments were chosen based on the condition that B/H considerations for WOE had been explicitly addressed, consistent with the analysis in the MOA/HR framework for several potential MOA(s) for carcinogenicity. The focus here was not on the conclusions of the assessments; rather, the extensive review and synthesis of data therein provided the opportunity to address the potential utility of comparative analysis based on the evolved B/H considerations for WOE in MOA/HR analysis. As such, the evidence and conclusions were not re-evaluated but were simply extracted from the referenced assessments and summarized in the narrative tables presented (Tables 5a,b and 6) for the purpose of illustrating the methodology. Similarly, assessment of the underlying investigations was not considered, though based on the approach presented here, this might constitute an important next step. The literature reviews were also not updated, as the current analysis does not focus on particular chemicals but rather the potential value of the proposed methodology.

Carbon Tetrachloride

This analysis is based on a published hazard and dose–response assessment for carbon tetrachloride (USEPA, 2010). Carbon

Table 5. (a) Comparative weight of evidence analysis for carbon tetrachloride: cytotoxic MOA[a]

Evolved Bradford Hill considerations		Supporting data	Inconsistent data	Missing data
1. Biological concordance		Sustained cytotoxicity and proliferation is a well-established MOA for chemically mediated carcinogenicity.		
2. Essentiality of key events		No carbon tetrachloride induced liver toxicity in CYP2E1 knockout mice. CYP450 inhibitors prevent carbon tetrachloride liver damage. Mice treated with CYP450 inducers have increased carbon tetrachloride toxicity in subchronic and chronic studies.		
3. Concordance of empirical observations	Dose–response	Cytotoxicty and proliferation are observed at doses equal to or lower than doses at which tumors develop in rats and male mice	Tumors elevated at the lowest dose tested in female mice (5 ppm) without hepatocellular damage.	
	Temporality	Progression from cytotoxicity to hepatocellular proliferation is supported in acute and subchronic studies in rodents. Temporal relationship of cytotoxicity, repair, proliferation and tumor development is also supported in chronic cancer bioassay in rats.		Temporal relationship in female mice is not clearly defined.
4. Consistency	Incidence	Hepatic toxicity, necrosis and regenerative proliferation have generally been reported in animals exposed to carbon tetrachloride orally or by inhalation and are correlated with CYP450 content. Some evidence of DNA damage observed in concert with cytotoxicity.	One study reported development of tumors in mice at doses that did not produce necrosis but design of study may have influenced this result as animals were killed 1 month after last treatment.	
5. Analogy				

MOA, mode of action.

[a]All conclusions in the above tables were extracted from the original US EPA toxicology review on carbon tetrachloride (USEPA, 2010).

(b) Comparative weight of evidence analysis for carbon tetrachloride: mutagenic MOA[a]

1. Biological concordance		Genotoxic MOA is well established for chemically mediated carcinogenicity.		
2. Essentiality of key events				
3. Concordance of empirical observations	Dose–response		Genotoxicity generally found at doses with cytotoxic effects.	Measurement of genetic damage to DNA has not been well

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		characterized at dose levels that do not cause cytotoxicity.
Temporality	Temporality not observed. Genotoxicity generally found in concert with cytotoxicity.	
Incidence	Extensive *in vitro* and *in vivo* genotoxic data are primarily negative. Doses where cytotoxic events are observed are lower than doses for which mutagenicity has been evaluated.	Genetically damaging events occurring at or below doses that induce cytotoxicity in laboratory rodents.
4. Consistency	Limited positive results in genotoxicity assays appear more related to a cytotoxic response than to a mutation event	
5. Analogy		

MOA, mode of action.
[a]All conclusions in the above tables were extracted from the original US EPA toxicology review on carbon tetrachloride (USEPA, 2010).

tetrachloride caused hepatocellular adenomas and carcinomas in rats, mice and hamsters in oral studies and in rats and mice following inhalation exposure. In addition to liver tumors, adrenal pheochromocytomas were observed in male and female mice following oral and inhalation exposure, for which it was concluded that data were inadequate to evaluate MOA. There was no increase in pheochromoctyomas in rats.

Based on the analysis of available data, including that on MOA, it was concluded in the assessment (USEPA, 2010) that the agent is likely a human carcinogen. Further, a potential MOA for carbon tetrachloride-induced liver tumors was hypothesized, with the following key events that included: (1) metabolism to the trichloromethyl radical by CYP2E1 and subsequent formation of the trichloromethylperoxy radical; (2) radical-induced damage leading to hepatocellular toxicity; and (3) sustained regenerative and proliferative changes in the liver in response to hepatotoxicity. The possibility that carbon tetrachloride may act via a mutagenic MOA (i.e., where mutation is an influential early key event in the induction of tumous versus, for example, being secondary to tissue damage) was also considered but not evaluated in a manner based on WOE considerations consistent with the MOA/HR framework. Based on the inconsistencies in the database supporting a potential role for the cytotoxicity, regenerative, proliferation-based MOA at the low end of the experimental exposure range and the complexity of the genotoxicity database, it was concluded that, "... the carcinogenic MOA for carbon tetrachloride is not known. Therefore, consistent with the *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005b), linear low-dose extrapolation as a default approach was applied to data for liver tumors and pheochromocytomas" (USEPA, 2010).

1,2,3-Trichloropropane

This analysis is based on a hazard and dose–response assessment of TCP released in 2009 (USEPA, 2009). Based on the observed statistically significant dose-related increases in multiple tumor types in both sexes of rats and mice in a 2-year carcinogenicity assessment (NTP, 1993) and related mechanistic data (including that on genotoxicity), it was concluded that TCP is "likely to be carcinogenic to humans" via a mutagenic MOA. Relevant data for alternative MOA(s) such as cytotoxicity with tissue repair and disruption of cell signaling were considered insufficient to evaluate. It was further concluded that the available data support a hypothesized mutagenic MOA with two key events: (1) metabolism to a DNA-reactive compound, and (2) (early) induction of mutations. A low-dose linear extrapolation approach to dose–response analysis was applied, consistent with the *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005b)

Comparative Weight of Evidence Analysis

Narrative comparative WOE summary tables were constructed for the hypothesized and alternative MOA(s) for carbon tetrachloride (Table 5a,b) and for a mutagenic MOA for TCP (Table 6) based on the consideration and evaluation of the data in the existing assessments (USEPA, 2009, 2010). For each postulated MOA, supporting data, inconsistent data and missing information were tabulated in the context of the evolved B/H considerations. As per MOA/HR framework recommendations, the information in the supporting and inconsistent data columns capture what has been observed, not what might be possible if more experiments had been performed. In addition, the

Table 6. Comparative weight of evidence analysis for 1,2,3-trichloropropane: mutagenic MOA

Evolved Bradford Hill considerations		Supporting data[a]	Inconsistent data[a]	Missing data[b]
1. Biological concordance		Genotoxic MOA is well established for chemically mediated carcinogenicity		
2. Essentiality of key events		Inducers/inhibitors of metabolism alter amount of DNA binding		Evidence for adduct conversion to genetic damage
3. Concordance of empirical observation	Dose–response	Dose-related formation of DNA-reactive metabolite, DNA adduct formation, tumor formation and time to tumor.		
	Temporality	Metabolism to reactive intermediate occurs within hours of exposure, adducts appear within hours and days of exposure, and tumors first appear after ≈ 9 months.		
	Incidence			No data to assess whether adduct formation frequency different from tumor frequency.
4. Consistency		Mutagenic effects *in vitro* accompanied by limited evidence of *in vivo* mutagenicity.	Adducts occur in tissues where no neoplastic effects were reported (spleen, liver and glandular stomach). Negative results from *in vivo* genotoxicity assessments (dominant lethal and micronucleus).	
5. Analogy		Other halogenated aliphatic chemicals (1,2,-dibromoethane and 1,2-dibromo-3-chloropropane) are mutagenic carcinogens. Other genotoxic chemicals are multisite and multispecies carcinogens.		

MOA, mode of action.

[a]All conclusions in the above tables were extracted from the original US EPA toxicology review on 1,2,3-trichloropropane (USEPA, 2009).

[b]The IRIS assessment did not comment on missing data; the information here represents the authors' views.

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information noted in the missing column only includes that which is testable and important for informing the MOA (i.e., critical data needs). Ideally, a discussion on whether the missing information is critical and would detract from or impact conclusions regarding the proposed MOA should accompany this comparative WOE table. Blank cells would typically represent instances where data either do not exist or are inadequate for evaluation. However, in this case, as the analysis draws upon an existing assessment, blank cells may also represent where text was either absent or inadequate to address the evolved B/H considerations.

Qualitative Assessment of Overall Evidence

For both case studies, the focus is not to conclude on the sufficiency of underlying data to support a particular MOA conclusion, but rather to illustrate the utility of the comparative WOE approach for increasing transparency in the assimilation of data.

Visually, Tables 5(a,b) and 6 highlight the availability of supporting and discrepant data on the MOA(s) evaluated for carbon tetrachloride and TCP. Comparative WOE analysis, for the two hypothesized MOA(s) for carbon tetrachloride based on the published assessment (USEPA, 2010), indicates that the supporting data for the hypothesized MOA involving cytotoxicity (necessarily within the range of experimental observation) fulfill a number of the evolved B/H considerations. This contrasts with the comparatively more limited support for the hypothesized mutagenic MOA. This difference highlights:

(1) the potential utility of comparative analysis for assessing the WOE of alternative MOA(s) for individual chemicals, based on the evolved B/H considerations to more explicitly indicate the degree of confidence in a particular MOA, and
(2) the desirability, in the interest of transparency and consistency, of separating conclusions reflecting assessment of the relative WOE for MOA in the observable experimental range based on articulated and explicit considerations from those based on inference or extrapolation to the low-dose range. It is anticipated that such an approach has the potential to increase transparency in delineating science judgment determinations from those related to public policy.

The comparative WOE analysis for TCP also provides a basis for comparison across chemicals of a relatively strong database for a mutagenic MOA, which can be contrasted with one that is relatively weak, potentially as a basis to increase consistency in determinations. In this case, perspective on the degree of confidence in the supporting WOE for the hypothesized mutagenic MOA for carbon tetrachloride (Table 5b) can be gained through comparison with the nature and extent of data available for the stronger database for TCP (Table 6).

Discussion

Comparative aspects of WOE analyses are illustrated here as a basis to contribute to transparency and consistency in delineating confidence/uncertainty in MOA/HR analysis based on the BH considerations. As noted by Guyton *et al.* (2008), Hill's (1965) considerations were not developed originally for evaluation of experimental/mechanistic data, though their utility for application in modified form to assess WOE in MOA analysis has been repeatedly though inconsistently tested. Based on increasing experience internationally in MOA/HR analysis (see, for example, Boobis *et al.*, 2006, 2008, Meek *et al.*, 2014), evolved B/H considerations are proposed here and clarified through delineation of the specific aspects addressed by each as framed by a series of questions. Definitions for these considerations have been additionally simplified and tailored to application in MOA analysis. The evolved B/H considerations were also rank ordered to reflect their relative contribution to WOE determinations and their utility exemplified in a comparative WOE approach.

The evolved B/H considerations build on previously published iterations and reflect experience in the application of MOA analysis. Several terms were clarified to facilitate assimilation of relevant chemical specific and biological data (i.e., "specificity" is now termed "essentiality of key events," "biological plausibility and coherence" is now termed "biological concordance" and concordance of empirical observations among key events delineated). In addition, considerations with limited relevance for evaluating MOA data (i.e., "strength," "coherence" and "experiment") were eliminated while other considerations (i.e., "analogy" and "incidence concordance") were added based on evolving experience with larger numbers of chemicals. It is hoped this evolved terminology, which reflects more common understanding within the broader risk assessment (versus epidemiological) community, will additionally contribute to consistency of use in MOA analysis. Finally, considerations were redefined as a basis to promote consistency and utility. For example, in publications of the IPCS MOA/HR framework (Boobis *et al.*, 2006, 2008; Sonich-Mullin *et al.*, 2001), consistency is defined as repeatability of key events in different studies; while in the USEPA cancer guidelines, consistency refers to the pattern of elevated risk observed across several independent studies (USEPA, 2005b). Neither definition accurately reflects the use of consistency in evaluating the WOE for hypothesized MOA(s). The former simply assesses reproducibility of results and, as such, may only contribute to the level of confidence in the occurrence of one key event. The latter definition is more appropriate to the assessment of the reproducibility of results in epidemiological and not mechanistic data sets. Consistency in the context of the MOA/HR framework more appropriately relates to evaluation of the WOE supporting interdependence of the key and end (adverse) events. Therefore, consistency was redefined here to reflect support of the pattern of effects across species/strains/organs and test systems for the hypothesized MOA. For example, if metabolism is a hypothesized key event in a carcinogenic MOA, the pattern of species-, strain- and sex-related variations in tumor response is compared to that expected based on known differences in metabolic profiles in the test systems. As such, it is not as important to assess if the occurrence of tumors is reproducible across studies, but rather, if the presence or absence of tumors in various species and strains is consistent with the hypothesized MOA.

Comparative WOE analysis is illustrated as a means of increasing understanding of the nature of transparency that is essential when evaluating confidence in the supporting WOE for hypothesized (potentially competing) MOAs. In doing so, it also provides a basis for increasing consistency in evaluation. Presentation of an overview of the data in a comparative manner (i.e., as supporting, inconsistent and missing) based on templates that cue evaluators concerning critical aspects provides concise insight into the extent of available data and relevant patterns in the existing database, which support various levels of confidence in considered options. In addition, this presentation concisely communicates areas of uncertainty (inconsistent data column and blank cells) and highlights areas of greatest impact for future research (missing data column). Ideally, further transparency on

the impact of this information (i.e. supporting, inconsistent and missing data) on the MOA conclusions would be provided in a detailed, supplemental discussion.

Synthesis of a collective data set to evaluate WOE for a hypothesized MOA is complex and challenging, requiring multidisciplinary input from both the research and risk assessment communities. This analysis is dependent upon transparent and consistent evaluation of the extent and nature of both chemical-specific and biological data versus supposition about possibilities for which there is essentially no experimental support. Characterization of the evolved B/H considerations is anticipated to contribute to more robust and transparent analyses, as a basis also to discourage, without clear rationale, the discounting of well-supported options based on the emphasis of outlying data of lesser quality.

This manuscript extends MOA/HR assessment through evolution of the B/H considerations and illustration of a comparative WOE analysis. Ultimately, it is anticipated that the additionally articulated and comparative aspects, which build on considerable recent experience in MOA analysis, will contribute to increasing transparency, consistency and methodological rigor in separating aspects of science judgment (i.e., weighting of options based on transparent consideration of available scientific support) from those of public policy in regulatory risk assessment (the latter of which sometimes involves embedded conservatism, to increase public health protection).

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Conflict of Interest

Several of the authors (C.M.P., A.N.B., C.M.N. and R.J.L) are employed by a subsidiary of Exxon Mobil, who produces materials evaluated by the US EPA. Methodological aspects based on case studies considered here do not relate to specific evaluations of relevance to Exxon Mobil.

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References

Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek ME, Vickers C, Willcocks D, Farland W. 2006. IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**: 781–792.

Boobis AR, Doe JE, Heinrich-Hirsche B, Meek ME, Munn S, Ruchirawat M, Schlatter J, Seed J, Vickers C. 2008. IPCS framework for analyzing the relevance of a non-cancer mode of action for humans. *Crit. Rev. Toxicol.* **38**: 87–96.

Borgert CJ, Mihairch EM, Ortego LS, Bentley KS, Holmes CM, Levine SL, Becker RA. 2011. Hypothesis-driven weight of evidence framework for evaluating data within the US EPA's Endocrine Disruptor Screening Program. *Regul. Toxicol. Pharmacol.* **61**(2): 185–191.

Carmichael N, Bausen M, Boobis AR, Cohen SM, Embry M, Fruijtier-Polloth C, Greim H, Lewis RJ, Meek ME, Mellor H, Vickers C, Doe F. 2011. Using mode of action information to improve regulatory decision-making: an ECETOC/ILSI RF/HESI workshop overview. *Crit. Rev. Toxicol.* **41**: 175–186.

Dellarco VL, Baetcke K. 2005. A risk assessment perspective: application of mode of action and human relevance frameworks to the analysis of rodent tumor data. *Toxicol. Sci.* **86**: 1–3.

Guyton KZ, Barone S, Brown RC, Euling SY, Jinot J, Makris S. 2008. Mode of action frameworks: a critical analysis. *J. Toxicol. Environ. Health B* **11**: 16–31.

Hill AB. 1965. The environment and disease: An association of causation. *Proc. R. Soc. Med.* **58**: 295–300.

Manibusan MK, Odin M, Eastmond DA. 2007. Postulated carbon tetrachloride mode of action: a review. *J. Environ. Sci. Health.* **25**(3): 189–209.

Meek ME. 2008. Recent developments in frameworks to consider human relevance of hypothesized modes of action for tumours in animals. *Environ. Mol. Mutagen.* **49**(2): 110–116.

Meek ME, Klaunig J. 2010. Interpreting available data and identifying critical data gaps for benzene risk assessment. *Chem. Biol. Interact.* **184**, 279–285.

Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD, Longfellow DG, Pastoor T, Seed J, Patton DD. 2003. A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **2003**(33): 591–653.

Meek ME, Berry C, Boobis AR, Cohen SM, Hartley M, Munn S, Olin SS, Schlatter J, Vickers C. 2008. Mode of action frameworks: a critical analysis. *J. Toxicol. Environ. Health B* **11**: 681–685.

Meek ME, Boobis A, Cote I, Dellarco V, Fotakis G, Munn S, Seed J, Vickers C. 2014. New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. *J. Appl. Toxicol.* **34**: 1–18.

NTP. 1993. Toxicology and carcinogenesis studies of 1,2,3-trichloropropane (CAS No. 96-18-4) in F344/N/N rats and B6C3F1 mice (gavage studies). Public Health Service, U.S. Department of Health and Human Services; NTP TR 384. NIH Publication No. 94–2839.National Institute of Enviornmental Health Sciences: Research Triangle Park, NC.

SAB. 1999. Minutes of the Chloroform Risk Assessment Review Subcommittee Meeting, October 27–28, 1999.

SAB. 2007. Subject: Advisory on EPA's assessments of carcinogenic effects of organic and inorganic arsenic: a report of the US EPA Science Advisory Board. Available from: http://www.yosemite.epa.gov/sab/sabproduct.nsf/EPA-SAB-07-008.pdf.

SAP. 2000. Atrazine: Hazard and dose–response assessment and characterization, SAP Report No. 2000–05. FIFRA Scientific Advisory Panel Meeting, June 27–29, 2000. Available from: http://www.epa.gov/scipoly/sap/meetings/2000/june27/finalatrazine.pdf.

Seed J, Carney E, Corley ER, Crofton K, DeSesso J, Foster P, Kavlock R, Kimmel G, Klaunig J, Meeik E, Preston J, Slikker W, Tabacova S, Williams G. 2005. Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Crit. Rev. Toxicol.* **35**: 663–672.

Sonich-Mullin C, Fielder R, Wiltse J, Baetcke K, Dempsey J, Fenner-Crisp P, Grant D, Hartley M, Knaap A, Kroese D, Mangelsdorf I, Meek ME, Rice J, Younes M. 2001. IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul. Toxicol. Pharmacol.* **34**: 146–152.

USEPA. 2005a. Science Issue Paper: Mode of carcinogenic action for cacodylic acid(dimethylarsinic acid, DMAV) and recommendations for dose response extrapolation July 26, 2005, Health Effects Division, Office of Pestcide Programs, US Environmental Protection Agency. Available from: http://www.epa.gov/oppsrrd1/reregistration/cacodylic_acid/dma_moa.pdf.

USEPA. 2005b. Guidelines for Carcinogen Risk Assessment, EPA/630/P-03/001 F. Risk Assessment Forum, US Environmental Protection Agency, Washington DC, March 2005.

USEPA. 2009. Toxicological Review of 1,2,3-Trichloropropane (CAS No. 96-18-4) in Support of Summary Information on the Integrated Risk Information System (IRIS), EPA 635/R-08/010 F, US Environmental Protection Agency, Washington DC, September 2009.

USEPA. 2010. Toxicological Review of Carbon Tetrachloride (CAS No. 56-23-5) in Support of Summary Information on the Integrated Risk Information System (IRIS), EPA 635/R-08/005F, US Environmental Protection Agency, Washington DC, March 2010.